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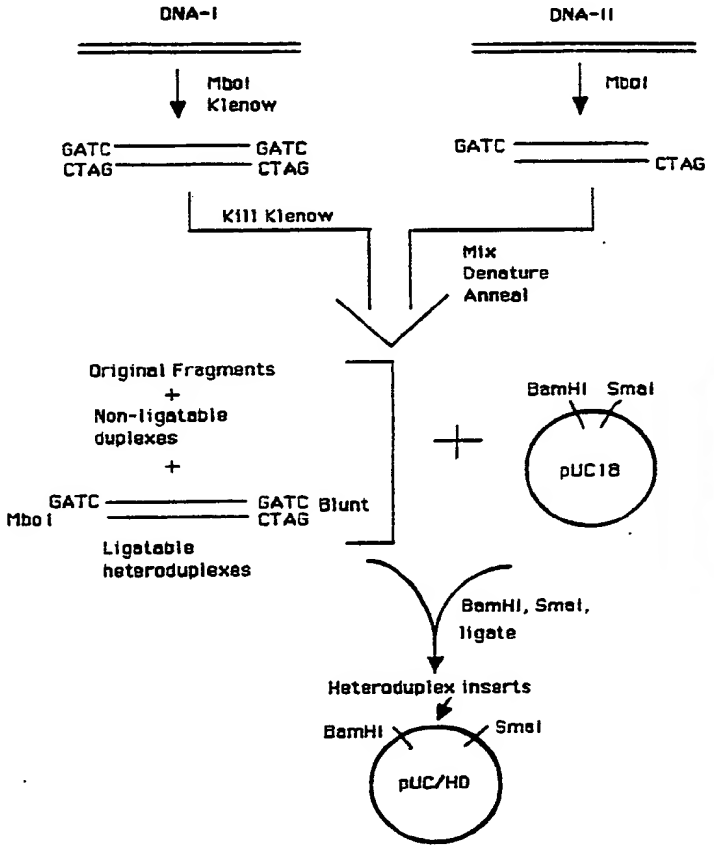
(57) Abstract

A method of obtaining common-sequence DNA fragments from two fragment mixtures, such as the fragments obtained from two different genomes, or from two different fragment-separation regions on a gel. The fragments of at least one of the two mixtures are modified such that heteroduplex fragments containing one strand derived from the first-mixture fragments and an opposite strand derived from homologous fragments in the second mixture can be isolated from homoduplexes formed by strand hybridization within each fragment mixture. The method can be used in applications relating to gene mapping, gene isolation, chromosome construction, cloning of conserved genes, and removal of repeat sequences from genomic DNA. Also disclosed are coincidence-sequence libraries formed by the method.



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COINCIDENCE CLONING METHOD AND LIBRARY

1. Field of the Invention

The present invention relates to methods for obtaining DNA sequences which are common to two DNA fragment mixtures derived from different sources, and to uses of the method for gene mapping and cloning.

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3. Background of the Invention

Recent developments in genetic mapping and cloning have created a need for additional methods for identifying and isolating genetic sequences from chromosomes and chromosome regions of interest. Such genetic sequences may then be used, for example, in identifying the genes responsible for genetic diseases.

Methods provided in the prior art allow for the isolation of genetic sequences from chromosomes and chromosome regions of interest in certain circumstances. Sorted chromosomes, isolated by physical methods from various cell types, and cloned sequence libraries prepared from sorted chromosomes, many of which are commercially available (American Type Culture Collection, Rockville, MD) contain genetic material from a selected chromosome, and are available for most, although not all, human chromosomes. While such sorted chromosomes have been valuable in providing genetic sequences for regions of interest in many cases, they do have some important limitations. One is a relatively high level of contamination with nonspecific genetic material, which decreases the utility of sorted chromosome material in isolating sequences of interest. Another is that because the basis of selection is at the level of the whole chromosome, it is difficult to focus down on specific regions of interest. This is in general true for tranlocations as well as wild type chromosomes in the absence of any method for specifically identifying and isolating the coincident sequences between two sources of genetic material.

Subtractive hybridization techniques have proven to be very valuable in isolating target genetic sequences present in only one of two sources. This is useful, for example, in isolating mRNAs (or the corresponding cDNAs)

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which are expressed in various cell types after activation or other stimuli. These methods rely on the use of two cell sources which are largely identical, only one of which contains the sequence of interest. Most often these are mRNAs or the corresponding cDNAs, although genomic DNA may also be used. Furthermore, these methods rely on the use of an excess of sequences from the source which does not contain the sequence of interest, in order to drive the hybridization reaction towards the formation of heteroduplexes.

4. Summary of the Invention

It is one general object of the invention to provide a method which allows the cloning and identification of DNA sequences which are common between different sources of DNA fragments.

It is another general object of the invention to provide such a method which substantially overcomes problems and limitations associated with the prior art, as discussed above.

Another object of the invention is to provide a variety of techniques which can be used to obtain such common sequences, according to the method of the invention.

Still another object of the invention is to use such method to advance or solve problems in several areas related to gene mapping, gene isolation, chromosome construction, and identification of large groups of conserved genes in humans and other mammalian species.

Providing a novel method for removing repeated sequences in a mixture of genomic fragments is yet another object of the invention.

The method of the invention is designed for obtaining from a first mixture of DNA duplex fragments derived from one source, those fragments which are homologous to and end hybridizable with the duplex DNA

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fragments in a second mixture of DNA fragments derived from another source. According to the method, the fragments are generated in a manner which allows heteroduplex, end-hybridized fragments formed by the hybridization of homologous DNA strands from the two DNA fragment mixtures to be isolated from homoduplex fragments produced by hybridization between opposite strands of the fragments in the first or second mixture only, and from heteroduplex fragments which are not end-hybridized.

Denatured strands from the fragments of the first and second mixtures are reacted in a reaction mix under hybridization conditions which yield heteroduplex, as well a homoduplex, reaction fragments, and the end-hybridized heteroduplex fragments are isolated from other nucleic acid species contained in the reaction mix. The hybridization reaction may be carried out with a molar ratio of the two fragment mixtore or with a molar excess of one of the mixtures.

In one general embodiment, the fragments are generated in such a way that when the paired strands forming the homoduplex fragments are mixed, denatured and reannealed, end-hybridized heteroduplex fragments can be isolated from other hybridization products by cloning, based on a unique pair of ligatable ends in the desired fragments. This method produces a library of cloned, coincident sequences which are enriched for single-copy sequences, since the heteroduplex fragments with non-hybridized ends are formed largely from repeated sequences. The ligatable fragment ends in the end-hybridized heteroduplex fragments may be generated either when the fragments are produced, by restriction endonuclease digestion, or by attachment of different linkers to the two sets of DNA fragments. The linkers may additionally contain methylated sites which allow generation of unique end pairs in heteroduplex fragments, by cutting with corresponding restriction endonucleases.

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In another embodiment, one of the fragment mixtures is modified with a label which allows physical separation of heteroduplex fragments from homoduplexes. The label may be an affinity label, such as biotin, which allows separation of heteroduplex species based on (a) initial binding to an affinity column and (b) subsequent release of the unlabeled strand of the heteroduplex by duplex denaturation. Alternatively, the label may be a density label which permits physical separation of heteroduplex from homoduplex strands based on density gradient centrifugation.

In still another embodiment, the fragments in the two mixtures are cloned in a vector which allows expression of one fragment strand or its transcript from one mixture, and the opposite fragment strand or its transcript from the other mixture. Separation of the heteroduplexes in this procedure is based on duplex formation and separation, for example, on a hydroxylapatite column.

Considering the various applications of the invention, one method of use is for cloning and/or analyzing the gene sequences, and preferably the single-copy sequences, which are carried on defined chromosomes or chromosome regions. Here, the sources of the two DNA fragment mixtures may be a two-species cell hybrid containing the specified chromosome from one species, such as a human/hamster hybrid containing a specified human chromosome, and a cell line from the one species, such as a human cell line. The procedure yields cloned, preferably single-copy, sequences present only in the defined chromosome.

Another important application of the method is for obtaining clones derived from a DNA fragment contained in a mixture of fragments, such as are typically obtained when DNA fragments are subfractionated, as by gel electrophoresis. As an example, partial digest fragments

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of genomic DNA, when fractionated by pulse field gel electrophoresis, will yield several band regions containing a gene region of interest, as evidenced by the binding of a selected probe to each of the regions of interest. After eluting the fragments from each of two such gel regions, these are then hybridized, according to the method of the invention, to produce common-sequence heteroduplex fragments derived from the desired probe-binding fragments on the gel.

The method may also be used for identifying and cloning gene sequences which are homologous between two different species, e.g., humans and another primate species. Because such homologous genes would be highly conserved, they are likely to represent gene functions which are important to the organism, such as gene functions related to immunological defenses, peptide hormones, and the like.

Another application of the method is for identifying and cloning specific chromosomal regions, such as the telomere regions at the end of chromosomes which appear to be required for chromosome stability. The method here involves cloning the coincident gene sequences from hybrid cells, each of which contains the chromosomal region of interest.

The method can also be used to enrich a mixture of genomic DNA fragments for single-copy sequences, either applied to a single DNA fragment mixture, such as total genomic fragments from a given source, or in conjunction with other applications mentioned above, in which the coincident fragments isolated by the method are enriched for single-copy sequences.

In another aspect, the invention includes a library of cloned DNA sequences produced by treating two DNA fragment mixtures according to the method of the invention, where the end-hybridized heterologous fragments are cloned into a suitable cloning vector.

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These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 is illustrates the method of the invention, wherein heterologous duplex fragments are isolated from homologous fragments on the basis of different fragment ends present in the heteroduplexes;

Figure 2 illustrates the method in another embodiment wherein fragments in the two fragments are equipped with different linkers, and heteroduplex fragments are selected on the basis of different restriction sites formed by the two linkers at the opposite fragment ends;

Figure 3 illustrates the method in another embodiment, in which the original homoduplex fragments are methylated at one of two different restriction sites, and heteroduplex fragments are isolated on the basis of unique opposite-end restriction sites after digestion with endonucleases corresponding to the two methylase sites;

Figure 4 shows the method in a related embodiment, in which linkers attached to each of the mixtures of fragments contain two common internal restriction sites, one of which is methylated, and different end sites, and heteroduplexes are distinguished from homoduplexes on the basis of different end sites which result after digestion with endonucleases specific for the internal linker sites;

Figure 5 illustrates another general embodiment of the method, in which heteroduplex molecules are isolated on the basis of binding to an affinity column and release of one strand of the heteroduplex on denaturation, where the released single-strand is contained in a cloning

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vector which can be readily converted to double-strand form;

Figure 6 illustrates an embodiment which is similar to that in Figure 5, but where the released single-strand material is annealed to form duplex fragments which can be cloned into a suitable cloning vector;

Figure 7 illustrates the method of the invention as it can be practiced using density-gradient centrifugation to separate heteroduplex from homoduplex fragments;

Figure 8 illustrates another method for carrying out the method in which heteroduplexes are separated from non-hybridizing DNA species by hydroxylapatite;

Figure 9 shows the steps in the application of the invention to isolating clones from single fragments obtained from a gel band region; and

Figure 10 illustrates the application of the method to isolating a human chromosome telomere.

Detailed Description of the Invention

I. Definitions

As used herein, the terms listed below have the following meaning:

(a) Homologous fragments: Two DNA duplex fragments are homologous with one another if the opposite strands in the two fragments are capable of forming stable duplex fragments under conditions of denaturation and reannealing.

(b) End-hybridizable: Two fragments are end-hybridizable if the homologous and opposite strands in the two fragments are capable of hybridizing with one another at their corresponding end regions by Watson-Crick base pairing. Such opposite strands are also said to be end-hybridizable.

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(c) End-hybridized: A fragment is end-hybridized if it is formed from end-hybridizable fragments. Typically, the strands forming an end-hybrized fragment will be hybridized along their entire lengths.

(d) Ligatable ends: The ends of a duplex fragments are ligatable if the fragment can be selectively incorporated into a cloning vector having defined ligation ends, in the presence of suitable ligation enzymes in vitro or in vivo. Ligatable ends include sticky ends, i.e., ends with short orverhang sequences capable of hybridizing with complementary overhang sequences, and blunt ends. Typically end-hybridized fragments will have ligatable ends.

(e) Homoduplex fragments: Homoduplex fragments are those formed by hybridization between homologous-fragment strands derived from the the same DNA fragment mixture.

(f) Hereroduplex fragments: Heteroduplex fragments are those formed by hybridization between homologous-fragment strands derived from different DNA fragment mixtures.

II. Preparing Coincidence-Clone Libraries

The method of the invention is aimed at obtaining gene sequences which are coincident in, i.e., common to the DNA fragments in two different mixtures of gene fragments. More particularly, the method is designed to obtain from the first mixture of duplex fragments, those fragments which are homologous to and end-hybridizable with the duplex fragments in the second fragment mixture.

These different mixtures can be obtained from different-species cells, from hybrid and non-hybrid cells, from different chromosomes or chromosome regions, from non-genomic sources, such as mitochondrial DNA, and in one embodiment, from the same DNA source, where the method is

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used to obtain predominantly single-copy gene sequences from the source. Various sources and methods of DNA isolation are detailed in Part A.

In preparing the DNA fragment mixtures, at least one of the mixtures is prepared in a manner such that when a strand from one mixture is hybridized with a homologous, end-hybridizable strand from the second mixture, the resulting end-hybridized heteroduplex fragment has properties which allow its separation from homoduplex fragments formed by hybridization between opposite strands of the fragments in the first or second mixtures only, and from duplex heteroduplex fragments which are not end-hypridized. Part D below describes embodiments of the method in which end-hybridized heteroduplex separation is based on unique fragment ends which allow cloning into a vector with selected insertion sites. In the embodiments covered in Part C, the methods of separating heteroduplex from homoduplex fragments involve physical separation of labeled fragments. Part C describes another general approach to the invention, in which heteroduplex separation is based on duplex formation from cloned, single-strand species.

A. Preparing DNA Fragment Mixtures

The mixture(s) of duplex DNA fragments used in the invention can be derived from a variety of multi-gene DNA source(s), such as the genomic DNA from eukaryotic cells or tissue samples, isolated chromosomes, mitochondrial DNA, and subfractions of DNA obtained by various DNA fragment separation procedures, such as gel electrophoresis or centrifugation methods. The actual source material used for DNA isolation may be whole cells, or subfractionations thereof, such as cell nuclei, or isolated chromosomes from cells.

In many applications, the cell line used as the DNA source for at least one of the fragment mixtures is a

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hybrid cell line containing at least one chromosome or chromosome region from one species, and the balance of the chromosome material from one or more other species. These hybrids may be obtained from known sources, or produced according to published methods. For example, Example 1 utilizes as one source of DNA material, the genomic DNA obtained from the somatic cell hybrid HHW661, a hamster-human hybrid containing a translocation of human chromosome region 4p onto hamster chromosome 5 (Wasmuth). In another application described in Section III below, the two sources of DNA are both hybrid cells, one containing a human chromosome 8, and another a human chromosome 4 with a translocated portion of human chromosome 8.

Where the source of DNA is a biological sample, the DNA can be isolated by standard procedures, which typically include successive phenol and phenol/chloroform extractions (Maniatis, p. 280). To illustrate, Example 1 describes the isolation of genomic DNA from two cell lines. Where the DNA mixtures are derived from subfractionated DNA fragments, such as from the agarose gels, conventional methods of DNA extraction, such as electroelution, gel maceration, or the like are used. The elution of DNA fragments from agarose gel regions is described in Example 9.

Typically, the isolated DNA is obtained in relatively intact form, and is fragmented by digestion with one or more selected restriction endonucleases, to form the desired mixture of DNA fragments. In the usual case, the DNA fragments in the mixture are formed by complete digestion with one or more endonucleases, to final fragment sizes of preferably between about 200 to 10,000 basepairs. Since in most applications, the heteroduplex fragments formed in the method are cloned, the upper size limit of fragments in the two mixtures is limited to clonable fragment sizes, generally less than 40 kilobases, and preferably no more than 10-20 kilobases.

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The choice of restriction endonucleases used in forming the DNA fragments in the two mixtures will depend on the specific approach used for isolating heteroduplex fragments, as will be clear from the various approaches described in Parts B-D below. In particular, the approach used will dictate optimal fragment size and nature of the cut ends. After endonuclease digestion to form the DNA fragments, the fragments may be further modified by filling recessed ends, ligation of end linkers and/or restriction-site methylation (Part B), by nucleotide labeling (Part C), and/or by cloning into a single-strand vector (Part D). Methods for performing such modifications are detailed in Examples 1-8 below.

B. Heteroduplex Selection By Cloning

This general embodiment exploits differences in end regions of the fragment hybridization products, to selectively clone end-hybridized heteroduplex fragments into a suitable cloning vector. In particular, the method is effective to isolate heteroduplexes consisting of end-hybridizable, homologous strands from homoduplex fragments and from duplex fragments which are not end-hybridized, i.e., which have one or more extended, non-hybridized end regions. Since the preponderance of duplex fragments which are not end-hybridized are formed by hybridization between repeat sequences, the method is therefore effective in enriching for single-copy sequences which are coincident to the two fragment mixtures.

One simple method of the invention which exploits end-hybridized heteroduplex selection by cloning is illustrated in Figure 1. Here two DNA fragment mixtures, designated DNA-I and DNA-II, are each prepared by digesting the corresponding DNA material to completion with a restriction endonuclease, such as MboI, which produces sticky fragment ends. One of these fragment mixtures, e.g., the DNA-I mixture, is further treated with

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Klenow fragment in the presence of the required nucleotides, to fill in both recessed ends of the fragments, forming blunt end fragments, as indicated.

The two fragment mixtures are now denatured and reacted under hybridization conditions which yield homoduplex and heteroduplex fragments. The hybridization reaction may be carried out by traditional hybridization methods, involving slow hybrid formation in a single-phase aqueous or aqueous/formamide medium, at a reaction temperature slightly below the melting temperature of the duplex material, according to published methods (Britten, 1968; Britten, 1985; Hames). Preferably, however, the hybridization reaction is performed according to a more recent phenol emulsion reaction technique (PERT), or formamide-phenol emulsion reaction technique (F-PERT), which greatly accelerates the hybridization reaction (Kohne, Casna). One potential drawback of the PERT hybridization approach in this method is the potential for DNA shearing during emulsification, resulting in blunt or sticky ends which are unrelated to the original fragment ends. This problem may be minimized by a light digestion with S1 nuclease after ligating the hybridization products into the cloning vector (below).

As shown in Figure 1, the hybridization reaction produces three general classes of duplex fragments. The first of these include original homoduplex fragments formed by hybridization between end-hybridizable homologous strands of the fragments in the first or second mixtures only. These homologous duplex fragments have either opposite blunt ends or opposite sticky ends. The second class of fragments are homoduplex or heteroduplex fragments formed from opposite strands which are not end-hybridizable. Typically such fragments are formed from imperfect copies of themselves, as is expected of repeat sequences contained in a variety of different-size digest fragments. At least one of the ends of these non-

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hybridized fragments is irregular in that it has a relatively long end-region of non-hybridized single-strand DNA. The third class of fragments are end-hybridized heteroduplex fragments. As seen in the mid portion of Figure 1, these fragments have opposite sticky and blunt ends.

The fragment mixture formed by reacting the opposite strands from the first and second DNA mixture are now cloned into a cloning vector which is designed to incorporate selectively only those duplex fragments having opposite sticky and blunt ends, i.e., heteroduplexes formed from end-hybridizable homologous strands. Typically, the vector is a plasmid, such as the pUC18 plasmid illustrated in Figure 1, which is cut at a polylinker site to expose ends which are compatible with the sticky and blunt ends of the desired heteroduplex fragments. The reaction fragments are ligated into the vector after removal of the small polylinker segment. Selection of successful recombinants, on a suitable host, is carried out by conventional methods. Since some of the end-hybridized heteroduplex fragments may be formed from end-hybridizable homologous strands, the successful recombinants may be further screened with labeled repeat sequence to eliminate the small percentage of repeat sequences.

This method is detailed in Example 1, which generally follows the reaction scheme shown in Figure 1. Three out of five clones which were screened were heterologous fragment inserts, i.e., derived from sequences common to both genomic DNA sources. Only one of the 48 clones which were screened by repeat-sequence probes showed evidence of repeat-sequence fragments.

In a related method, the two DNA fragment mixtures are prepared by (a) digesting the first and second DNA with different endonucleases, such that the first and second fragment mixtures have different sticky

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ends. The nucleases used are selected such that the hybrid sticky ends formed by hybridization between first- and second-mixture equal-size strands are different from either of the homoduplex sticky ends in the original fragment mixtures.

Figure 2 illustrates another approach for generating fragment mixtures in which homoduplex and end-hybridized heteroduplex fragments can be separated by an appropriate cloning vector. Here the characteristic sticky ends used to distinguish homoduplex from end-hybridized heteroduplex fragments are provided by end linkers which are attached to the original digest fragments. With reference to Figure 2, the DNA material from the two sources is originally digested to completion with an endonuclease, such as MboI, which preferably cuts the DNA at relatively frequent intervals, e.g., every 200-5,000 basepairs. The first fragment mixture is then mixed with one linker, designated linker I in the figure, which is designed for attachment to the fragment sticky end and provides an internal, preferably infrequent restriction site, such as the XhoI site indicated. Treatment of this fragment mixture with the linker-site endonuclease now yields relatively small fragments with opposite rare-cutter site ends. A second linker, similarly designed for attachment to the original digest mixture and carrying a second internal and preferably infrequent endonuclease site, such as NotI (linker II in Figure 2) is similarly attached to the second fragment mixture, which is then treated with the linker-site endonuclease, to generate a second fragment mixture composed of small fragments with infrequent-site sticky ends. In addition to the requirement of a selected restriction-site sequence, the linker sequences are also designed for hybridization with one another, as illustrated in linkers I and II in Figure 2.

The two fragment mixtures are now mixed, denatured, and reannealed, as above, to produce hybridized

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fragments consisting of end-hybridized and non-end-hybridized homoduplex and heteroduplex fragments. The end-hybridized homoduplex fragments have opposite sticky ends which are either both linker I or both linker II ends; non-end-hybridized homoduplex and heteroduplex fragments have at least one irregular end; and end-hybridized heteroduplex fragments have one linker I end and an opposite linker II end, as indicated. These fragments are mixed into a cloning vector which selectively incorporates the linkerI/linker II ends, and successful recombinants are selected as above.

In the scheme illustrated in Figure 2, and described in Example 2, the two fragment mixtures are prepared with XhoI and NotI sticky ends, and the hybridized fragments are cloned into the XhoI/NotI site of a Bluescript® plasmid.

Figure 3 illustrates another procedure for preparing the fragment mixtures for selection of heteroduplex fragments on the basis of hybridized-end characteristics. This procedure utilizes methylation at internal restriction sites, followed by endonuclease treatment of the hybridization products, to generate unique fragment ends in equal-size heteroduplexes.

The DNA fragment mixtures are initially prepared by complete digestion with a one or more selected endonucleases, where the endonuclease(s) used is selected to produce preferred fragment sizes of at least about 1,000-2,000 kilobases, to insure that most of the fragmenst contain internal frequent-cutting sequences, such as AluI and HaeIII sequences. For illustrative purposes, the fragments shown in Figure 3, which are produced by BamHI (B) digestion, contain a single internal AluI (A) and two HaeIII (H) restriction sites. The first fragment mixture, designated DNA-I in the figure, is treated with a selected methylase, such as AluI methylase, to methylate both fragment strands at one frequent-cutting

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site, as indicated by the "*" symbols in the figure. Similarly, the second fragment mixture is treated with a second methylase, such as HaeIII methylase, to methylate both fragment strands at a second frequent-cutting site in the strands.

The two fragment mixtures are mixed, denatured, and reannealed, as above, to produce hybridized fragments consisting of both homoduplex and heteroduplex fragments. With reference to Figure 3, it is seen that the homoduplex fragments (whether or not formed from end-hybridizable strands) are methylated at both strands at one frequent-cutting site only, and thus can be digested by endonuclease cutting at the non-methylated frequent-cutting site. By contrast, the heteroduplex fragments (again, whether or not formed from end-hybridizable strands) are methylated on one strand or the other at both frequent-cutting sites, and therefore protected against endonuclease digestion by endonucleases which require either frequent-cutting sequence.

Digestion of the homoduplex fragments with endonucleases, such as AluI and HaeIII, which cut at the two frequent-cutting sites, will cleave all homoduplex fragments (at the non-methylated frequent-cutting sites,) but leave the heteroduplex fragments intact. As a result, only those duplex fragments which (a) are formed of end-hybridizable strands, and are either (b) heteroduplex fragments or (c) homoduplex fragments which contain neither of the above frequent-cutting sites, will retain the original sticky ends, e.g., the two opposite BamHI ends, used in generating the two fragment mixtures.

Following digestion with the two frequent-cutting endonucleases, the fragments are cloned into a vector which selectively incorporates fragments with original sticky ends. Successful recombinants are selected as above. Clones which do not contain either of the two frequent-cutting sequences used above, and which

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are therefore suspect as being derived from homoduplex fragments, can be identified by resistance of the plasmids to linearization by digestion with either of the two endonucleases. Details of the method are given in Example 3.

A fourth method of heteroduplex selection by cloning employs elements of both the end-linker and site-methylation approaches just described. In this method, which is illustrated in Figure 4, fragment digestion and attachment of different linkers (linkers I and II in the figure) are carried out substantially as in the method illustrated in Figure 2. Here, however, the linkers contain, in addition to the "proximal" sticky end used for ligation to the fragments, such as an MboI sticky end, and a rare-cutting sequence near the "distal" linker end, such as a NotI sequence, two "internal" restriction sequences, in the present example, AluI, and HaeIII sites. The two internal-site sequences are referred to more generally as A and B sequences, and the distal-site sequences, such as NotI and XhoI sequences, as C and D sequences. Thus linker I in the figure has the sequences A/B/C and linker II, the sequences A/B/D.

Following linker attachment, the DNA-I fragment mixture, having the linker-I ends, is treated with a methylase which is specific for the A linker sequence, and the DNA-II fragment mixture, having the linker-II ends, is treated with a methylase specific for the B linker sequence. The resulting fragment mixtures are methylated at both linker strands, at either the A or B sequence and at any A or B internal sequences in the fragments, as indicated in the figure.

The two fragment mixtures prepared as above are now mixed, denatured and annealed, as above, to produce (a) end-hybridized homoduplex fragments which are protected at one or the other but not both of the A or B linker sequences, (b) non-end-hybridized homoduplex and

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heteroduplex fragments having at least one irregular end and (c) end-hybridized heteroduplex fragments which are protected at both A and B linker sequences, by virtue of different-strand methylation in the linker region, and having opposite-end C and D sequences. Digestion of the reaction fragments with endonucleases specific for both A and B sequences cuts the homoduplexes at all A or B sequences, producing fragments with either A-sequence or B-sequence opposite sticky ends. Heteroduplexes, by contrast, are not cut by either endonuclease, and thus retain their opposite C and D sequences. Further digestion with endonucleases specific for C and D sequences now produce C and D sticky ends in the opposite ends of end-hybridized heteroduplex fragments. It can be appreciated that a small percentage of fragments containing internal C or D sequences may have opposite C or opposite D sticky ends.

The digest fragments are now cloned into a suitable vector containing C and D sticky end sites, and the successful recombinants selected as above. The fragments may also be cloned into vectors containing opposed C-sequence sticky ends, or D-sequence sticky ends, to clone those heteroduplex fragments containing internal C or D sequences. Example 4 details a procedure which follows the general scheme shown in Figure 4.

As can be appreciated from the above, all of the procedures presented above share a number of common features and advantages:

(a) In all procedures, the two fragment mixtures are generated from the associated DNA source in such a way that the hybridization products produced by reacting the two fragment mixtures under hybridization conditions can be separated on the basis of selective incorporation into a suitable cloning vector. Thus the method for isolating the desired heteroduplex fragments also yields a fragment

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library which is enriched for end-hybridizable, coincident sequences.

(b) All of the procedures involve relatively simple digestion and methylation and/or linker attachment manipulations in generating the fragments.

(c) All of the procedures effectively select against repeat sequences, by virtue of the irregular fragment ends which are generally associated with repeat sequences.

C. Heteroduplex Selection Based on Physical Properties

In the various embodiments of the invention described in this section, heteroduplex fragments are separated from homoduplex fragments on the basis of a physical property related to a nucleotide label. The label may be either a density label, such as an ^{15}N-labeled nucleotide, or an affinity label, such as biotin, which is incorporated into both strands of one fragment mixture. Heteroduplex fragment separation then involves isolating fragments containing one labeled and one unlabeled strand from completely labeled or completely unlabeled homoduplex fragments.

An example of one approach using an affinity label for heteroduplex separation is illustrated in Figure 5. Here one fragment mixture, designated DNA-I, is labeled in both strands with biotin. The other mixture, designated DNA-II, is cloned into a vector, such as M13, which can be grown in single strand form. Because the cloning vector is used as a source of one strand only (either the sense or anti-sense strand), the original fragments are prepared by digestion with two endonucleases, such as EcoRI and HindIII, so that the fragments can be introduced directionally into the vector. The DNA-I fragments are prepared by digestion with the same pair of enzymes.

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The method illustrated in Figure 5 does not involve a cloning step which discriminates against unequal-size heteroduplex fragments. Therefore, in order to ensure that the heteroduplexes are predominantly end-hybridized, one of the two mixtures, and preferably the DNA-I fragment mixture, is initially treated to remove repeat sequences. This can be done by conventional slow hybridization techniques carried out in a single-phase reaction system, as referenced above. Typically, the denatured fragments in the mixture are hybridized to an initial $C_o t$ value at which most of the repeated sequences are hybridized, and most of the single-copy sequences are still in single-strand form. After removing the hybridized species by binding to a hydroxyapatite column, the single-strand material is carried to a second $C_o t$ value at which the single-copy strands are predominantly hybridized. This general techniques is detailed in Example 5A.

Alternatively, several methods described herein for selecting coincident species may also be applied initially to removing repeat sequences, as will be considered in Section III below.

The single-copy fragments from above are now labeled with a biotin label, according to one of the general procedures detailed in Example 5B. All of the methods produce labeling of both fragment strands, as is required. Although biotin is the preferred affinity label, any label which can be incorporated into polynucleotides and which has a binding partner capable of binding the label specifically and with high affinity may be used. The affinity label is also referred to herein as an epitopic molecule, and the binding partner, as a binding molecule. Exemplary binding pairs of epitopic molecule/binding molecule include biotin/avidin, biotin/streptavidin, antigen/antibody, and carbohydrate/lectin. The methods described in Example 5B for incorporation of biotinylated nucleotides into polynucleotide fragments are

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generally applicable to incorporation of nucleotides derivatized with other epitopic molecules.

The labeled, single-copy strands are now mixed with the cloning vector containing the DNA-II fragment inserts and grown under conditions which yield one vector strand (sense or anti-sense) only. The mixture is denatured and allowed to reanneal, as above. With reference to Figure 5, the annealing reaction produces homoduplex fragments, heteroduplex fragments consisting of a labeled fragment strand from the DNA-I mixture, and the homologous DNA strand from the cloned DNA-II mixture, and single-strand species from both mixtures (not shown). These reaction products are now applied to an affinity support material having surface-bound binding molecules, to bind all labeled duplex fragments to the support, with elution of non-hybridized DNA-II strands. The support-bound material is now denatured, either by heating, raising pH, and/or addition of denaturing solvents, such as a water/formamide mixture, to release the non-labeled, cloned, single strand material from the support. The resulting phage material is used to transfect a suitable bacterial host, and grown in either single-strand or double-strand form. This method is detailed in Example 5.

A related method which does not require removing repeat sequences from one of the fragment mixtures is illustrated in Figure 6. Here both fragment mixtures are generated by digestion with the same endonuclease, and one of the fragment mixtures is labeled, as indicated. The labeled and unlabeled mixtures are now mixed, denatured, and reannealed, as above, producing homoduplex fragments with both or neither fragments labeled, and heteroduplex fragments with one strand only labeled.

The hybridization products are passed through an avidin or streptavidin column, binding labeled homoduplex and heteroduplex fragments to the column, with elution of the unlabeled homoduplex fragments. The bound fragments

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are now denatured, as above, and the unlabeled single-strand species are eluted. It will be appreciated that the eluted DNA strands are (a) all derived from the unlabeled fragment mixture, (b) represent both end-hybridizable and non-end-hybridizable strands, and (c) include both sense and anti-sense strands. These single strand species are ethanol precipitated, and reannealed, forming homoduplex fragments which are derived from heteroduplex fragments only, i.e., are all coincident with fragments in the labeled fragment mixture.

With continued reference to the figure, the reannealed end-hybridized duplex fragments (representing predominantly single-copy fragments), contain the same sticky ends as the original unlabeled fragments, whereas the duplex fragments which are not end-hybridized contain at least one irregular end. The total fragments are mixed with a suitable cloning vector which selectively incorporates the regular sticky end fragments, with selection for successful recombinants as above. The method is detailed in Example 6, where the fragment mixtures are formed with MboI digestion, and the reannealed unlabeled fragments are cloned into the MboI site of a pUC18 vector.

Figure 7 illustrates a method of density gradient separation of heterologous and homologous fragments. Here the two fragment mixtures are prepared by digestion with a frequent-cutting endonuclease, such as MboI, and one of the fragment mixtures is labeled, as above, by incorporation of a heavy isotopic nucleotide, such as ^{15}N-labeled nucleotides, where the label may be carried in one or more of the nucleotide species. Incorporation of the labeled nucleotides is by one of the methods detailed in Example 5B for incorporation of biotinylated nucleotides into duplex DNA.

The labeled and unlabeled fragments are mixed, denatured and reannealed as above, yielding homoduplexes with both or neither unlabeled strands and coincident

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heteroduplex fragments with one labeled and one unlabeled strand. These three species of duplex fragments are then fractionated by equilibrium density centrifugation, according to classical procedures, such as on a CsCl gradient. In the density gradient shown in Figure 7, where the labeled strands are shown by wavy lines, the heteroduplex fragments fractionate between the lighter unlabeled homoduplexes and the heavier, fully labeled homoduplexes. The heteroduplex fraction is recovered by aspiration. This fraction contains both end-hybridized and non-end-hybridized fragments, and the former are isolated by cloning into an appropriate cloning site in a plasmid vector, as in the method immediately above. Details of this method are given in Example 7.

D. Coincidence Fragment Selection by Duplex Formation

The method presented in this section relies on the formation of duplex fragments from homologous sense and anti-sense strands contributed by the first and second DNA fragment mixtures, respectively. With reference to Figure 8, each of the two fragment mixtures is initially prepared by digestion with two selected endonucleases, such as EcoRI and HindIII, producing fragments which can be inserted in an oriented fashion in a cloning vector which can be grown in either a single-strand or double-strand form.

In a preferred approach, the two fragments mixtures are cloned into a pair of cloning vectors which are designed to receive fragments in one or two defined orientations, in a double-strand form, and which therefore produce opposite insert strands, in a single-strand form. One such vector pair includes the vectors M13/mp18 and M13/mp19 which have polylinkers arranged in opposite orientations, for accepting inserts cut with a pair of selected endonucleases, such as EcoRI and HindIII, in opposite orientations. The cloning step is shown in Figure

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8, where the first EcoRI/HindIII fragment mixture is cloned into an mp19 plasmid in one orientation, and the second EcoRI/HindIII fragment mixture is cloned into an mp18 plasmid in the opposite orientation. When these plasmids are grown under single-strand phage conditions, the mp19 vector produces the sense (+) strand of the insert, and the mp18 vector, the anti-sense (-) strand.

The two single-strand phage mixtures, containing the opposite-strand inserts, are now mixed and annealed, preferably using the above F-PERT procedure, yielding phage complexes having opposite strand duplex regions. As indicated in Figure 8, phage complexes formed from end-hybridizable inserts allow end region annealing of the opposite-strand polylinker sequences present in the two cloning vectors, so that the duplex inserts are bounded by defined duplex restriction sequences, and in particular, the sequences used for inserting the original fragments into the double-strand vectors. In the example illustrated in Figure 8, and detailed in Example 8, these sequences are those recognized by EcoRI and HindIII. By contrast, opposite strand complexes formed from non-end-hybridizable fragments have at least one irregular mismatch at the insert end which prevents annealing at the vector polylinker sequences.

The annealed fragments are now digested with endonucleases which cut at opposite vector polylinker sites, and preferably at the sites used to introduce the original fragments into the two vectors, to avoid cutting the inserts themselves at internal sites. Thus, in Figure 8, the EcoRI and HindIII sites used to generate the original fragment mixtures, and to introduce the fragment mixtures into the two cloning vectors, are also used to digest the duplex phage species. The resulting digest fragments are then cloned into a suitable cloning vector, such as pUC18 opened at its EcoRI and HindIII sites, which

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selectively incorporates the equal-size duplex fragments. This method is illustrated in Example 8.

This approach has the potential for greater discrimination between coincident and non-coincident sequences, since only coincident sequences form hybrid duplexes, and therefore could be introduced into a duplex cloning vector. The method also has the potential for good discrimination between end-hybridizable and non-end-hybridizable duplexes, since only equal-size duplexes are released from the hybridized products in a clonable form. The limitation of the method is the need for two cloning steps, one in forming the single-strand fragment mixtures, and the second in selecting single-copy annealed hybridization products.

III. Applications

This section discusses applications of the coincident cloning method to gene mapping, gene isolation, chromosome construction, cloning of conserved genomic sequences, and removing repeat sequences from genomic DNA.

A. Cloning Single-Copy Chromosome-Region Sequences

There are a variety of applications in which it is useful to identify and clone coincident single-copy sequences from different DNA sources. For example, it would be useful to clone all of the single-copy gene sequences from a given human chromosome or chromosome region. In many instances it is not possible to isolate the chromosome or chromosome region of interest, either because of limitations of physical isolation or because the chromosome region of interest is not mapped.

As one example of this application, assume the problem is to clone all of the single-copy sequences in human chromosome 4 (C4), for purposes of constructing a library of probes for C4. As a first step, one would first construct a human/non-human hybrid containing C4 on

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a background of non-human chromosomes. Typically the hybrid would be a mouse/human or hamster/human hybrid containing a single C4 chromosome. To clone the C4 single-copy sequences, restriction fragments from this source (DNA-I) and from the entire human genome (DNA-II) are mixed, and reacted under hybridization conditions, according to one of the methods from Section II above, to produce hybridization products representing coincident sequences, i.e., sequences associated with C4. The hybridization products are further treated and cloned, as above, to yield a library of cloned, C4 sequences enriched for single-copy species. These clones in turn can be radiolabeled to provide a substantially complete bank of probes for human C4. This application is illustrated particularly in Example 1, which demonstrates the cloning of single-copy species associated with human chromosome 5 containing a translocation of chromosome 4p.

It will be appreciated how the method can be similarly applied, in the construction and analysis of hybrid genomes, to answering questions about (a) how much of the total genome in a hybrid cell is contributed by a selected species, (b) which chromosome(s) or chromosome segment(s) are present from the selected species, and (c) changes in chromosome composition over time.

B. Cloning Sequences of Single Genomic Fragments

An important problem in human genetic studies is identifying genes or gene groups which are related to particular genetic diseases. Often the search for such genes begins by screening human single-copy probes, using restriction fragment polymorphism analysis to identify probes which are associated with a disease related restriction pattern. These probes presumably correspond to regions which are genetically linked to the disease-related gene of interest, but which may still be up to 1,000 kilobases or more from the gene of interest. Once

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these probes are identified, they may be used to probe large genomic digest fragments which can be size-fractionated by new gel electrophoresis methods, such as pulsed-field gel electrophoresis (PFGE), which provide greater resolution of large genomic fragments by virtue of orthogonally disposed electrical fields (Smith).

In theory, PFGE can be used to fractionate large genomic fractions, and the fragments of interest, i.e., those associated with one or more identified probes, can be identified on the gel by probe binding techniques, such as Southern blotting. The limitation of this approach is the relatively large number of same-size digest fragments which will typically be found in a probe-binding gel region. That is, elution of the digest fragments from a probe binding region may yield many distinct fragments, without any practical way of resolving and isolating the probe-binding fragment of interest. Accordingly, efforts to map the fragment region of interest with cloned library subfragments would be quite difficult, since most of the cloned subfragments would not relate to the fragment of interest.

The application of the present invention to this problem is illustrated in Figure 9. Here the duplex DNA shown at the top in the figure represents a segment of DNA containing a probe-binding region P which is adjacent a gene region of interest G where both P and G are located between a pair of restriction sites S_3/S_4. The restriction sites S_i are preferably at least about 100 kilobases from one another. The objective is to clone fragments in the S_3/S_4 fragment segment only, for purposes of further mapping the relationship between P and G and identifying one or more cloned G subfragments.

As a first step in the method, the DNA is partially digested with the endonuclease which cuts at the rare S sequences. Methods for forming partial DNA digests which are suitable in the present method are given in

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Example 9. As seen, the partial digest produces a number of different-size fragments which contain the desired S_3/S_4 segment, including the S_3/S_4 fragment. The partial digest fragments are now fractionated by PFGE, substantially according to methods described and referenced in Example 9, and the gel is examined for probe-binding regions (containing the S_3/S_4 fragment) by Southern blotting, using the previously selected probe. Two of the probe binding regions are now removed and the digest fragments are eluted. For purposes of illustration, it is assumed that the probe-binding regions identified as S_1/S_4 and S_3/S_4 are so identified and eluted. In particular, it is an advantage to select as one of the probe-binding region, the region containing the smallest probe-binding region, presumably representing the smallest digest fragment possible, in this case, the fragment S_3/S_4.

The two eluted gel fractions are used as the two DNA sources from which coincident sequences can be cloned, according to the method of the invention. Each of these fragment mixtures is digested to completion with one or two selected endonucleases and prepared for hybridization, according to one of the methods detailed in Section II. Hybridization and cloning of heteroduplex fragments formed from end-hybridized strands yield cloned subfragments which are common to both elutate mixtures. Assuming that the S_3/S_4 fragment contains the only sequences common to both eluates, the method yields clones containing only sequences present in the S_3/S_4 fragment, and enriches single-copy sequences. With this limited library, mapping of and gene identification in the S_3/S_4 fragment is greatly simplified.

It will be appreciated that the method is similarly applicable with other recombinants for generating fragments that fractionate in different parts of the gel, or in more than one gel, which contain coincident

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sequences. This may be accomplished, for example, by using a source or sources containing a restriction fragment-linked polymorphism for the rare cutter enzyme S in the region of interest, or by cutting with two different rare cutter enzymes.

C. Cloning Conserved Sequences

It is now recognized that many of the functional genes in higher organisms have been relatively conserved during evolution, as evidenced by considerable sequence homology between analogous-function genes in related organisms. In general, greater conservation is seen in more closely related species, and also with more important, i.e., fundamental gene products, such as histones and hemoglobin. Because conserved gene sequences are likely to represent the most important functional genes in an organism, it would be advantageous to obtain all of the conserved sequences of an organism, particularly in humans, in cloned form.

In practicing the method, two genomic sources from related species are selected. For cloning human conserved genes, a primate species such as lemur would be preferred, since a more closely related species, such as chimpanzee, may give too much general gene homology. The two DNA sources are fragmented, denatured, reannealed and cloned, according to one of the procedures in Section II, yielding a library of conserved sequences enriched for single-copy sequences.

D. Cloning Human Telomere Regions

To date, efforts to identify telomere sequences in human chromosomes have not been successful, despite the importance of this region for chromosome stability. Cloned telomere sequences may be important, for example, for constructing stable chromosomes which can be used for gene therapy.

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One approach to cloning telomere sequences, according to the present invention, is outlined in Figure 10. The upper portion of the figure shows a known translocation in the end regions of human chromosomes 4 and 8 in which end portions of the two chromosomes, including the telomere region, are exchanged. The objective of the method is to clone those sequences, presumably including telomeric sequences, which are present in the C8 translocation on the C4 chromosome.

As a first step in the method, hybrid cells containing in one case chromosome 8, and in the other case, chromosome 4 with the chromosome 8 translocation are produced. As an example, one hybrid cell is a Chinese hamster ovary (CHO) cell containing the C4/C8 translocation chromosome, and the other hybrid is a mouse cell containing a normal human C8 chromosome, as indicated in the figure. DNA from the two cell types is isolated, and fragmented as above, to form the two DNA fragment mixtures used in the method. The coincidence sequences, which include those single-copy sequences derived from the translocated portion of C8, as well as those sequences conserved between Chinese hamsters and mice, are obtained by one of the coincidence methods discussed in Section II. Those clones containing rodent conserved sequences are then identified and removed by screening with total DNA from either rodent cell.

E. Cloning Infectious Microorganisms

This application is aimed at cloning DNA sequences derived from infectious microorganisms which (a) have not yet been identified and isolated, and (b) are infectious toward disparate hosts, such as humans and rodents. The two infected cell types from the disparate hosts are used to produce the two DNA fragment mixtures from which coincident sequences will be derived, according to the method of the invention. The library of cloned

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sequences may be further screened with the sequences derived from the two host sequences, such as human and hamster genomic sequences, to remove host sequences from the library. The remaining cloned fragments now represent sequences derived from the infectious agent. These clones, in turn, can be used as probes for identifying the infection in cells, or for determining sequences in the genome in the infectious agent, for purposes of preparing diagnostic or vaccine reagents.

F. Enriching Genomic Fragments for Single-Copy Sequences

As indicated above, the method of the invention may also be used for removing repeat sequences from genomic DNA, to enrich a genomic fragment mixture for single-copy sequences. This application is based on the ability of the method to discriminate against heterologous fragments formed from non-end-hybridizable strands, associated predominantly with repeat sequence hybrids.

In practicing the method, the genomic material of interest is divided into two portions, and each of these is then used in generating the two fragment mixtures which are to be hybridized. The two mixtures are reacted under hybridization conditions which yield heteroduplex fragments, as discussed above, and these are further cloned to selectively remove fragments formed from non-end-hybridized fragments. The resulting genomic library can be further screened with known repeat sequences to further enrich the library for single-copy sequences.

From the foregoing, it can be appreciated how various objects of the invention are met. The method of the invention provides a simple, practical approach for selecting out of two large mixtures of genomic fragments, those coincident sequences which are common to both mixtures. In particular, the method typically yields a library of cloned coincident sequences which are enriched for single-copy sequences. The method may be performed by

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a variety of procedures which rely on fragment end characteristics, physical properties, and/or duplex formation in cloned single-strand form.

The method can be applied usefully to a number of significant problems in genetic mapping and gene cloning, including the specific applications described in this section.

The following examples illustrate methods of coincidence cloning using heteroduplex cloning and/or physical selection methods according to the invention, and applications of coincidence cloning to selection of specific genomic sequences. The examples are intended to illustrate, but not limit, the scope of the invention.

Materials and Methods

M13/mp18 and M13/mp19 are obtained from New England Biolabs (Beverly, MA). Cloning plasmid pUC18 and its host E. coli strain JM103 are obtained from Pharmacia. Bluescript® cloning vector containing NotI and XhoI cloning site is supplied by Stratagene (San Diego, CA).

Terminal transferase (calf thymus), alkaline phosphatase (calf intestine), polynucleotide kinase, Klenow reagent, and S1 nuclease are all obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); SP6 and T7 polymerase, from Promega Biotech (Madison, WI); and proteinase K, RNase and DNase, from Sigma (St. Louis, MO);

NotI, XhoI, SmaI, BamHI, HindIII, EcoRI, T4 DNA ligase and T4 DNA polymerase, SalI, HaeIII, AluI, NotI methylase, XhoI methylase, HaeIII methylase, and AluI methylase are obtained from New England Biolabs (Beverly, MA); oligo dT primer and oligo dA and oligo dT cellulose, from PL Biochemicals (Milwaukee, WI); Chelex-100, from Bio-Rad (Richmond, CA); Sephadex G-50, from Pharmacia (Piscataway, NJ); and streptavidin agarose, from Bethesda Research Labs (Bethesda, MD). Low-gelling temperature

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agarose is obtained from Sea Plaque, FMC, and proteinase K and phenylmethylsulfonyl fluoride (PMSF), from standard sources. Nitrocellulose filters are obtained from Schleicher and Schuell.

Synthetic oligonucleotides for vector modifications to introduce NotI and SfiI linkers are prepared by conventional phosphotriester methods (Duckworth) or the phosphoramidite method as reported (Beaucage; Matteucci), and can be prepared using commercially available automated oligonucleotide synthesizers. Alternatively, custom designed synthetic oligonucleotides may be purchased, for example, from Synthetic Genetics (San Diego, CA). Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles gamma-^{32}p-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 ug of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 ul of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate.

Incubation times of about one hour to two hours at about 37°C are workable, although variations can be easily tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol (70%). If desired, size separation of the cleaved

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fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow reagent) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20° to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 0.1-1.0 mM dNTPs. The Klenow fragment fills in at 5' single-stranded overhangs in the presence of the four nucleotides. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the overhang. After treatment with Klenow reagent, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portions of DNA. In particular, the nicking of 5' hairpins formed on synthesis of cDNA is achieved.

Ligations are performed in 15-50 ul volumes under the following standard conditions and temperatures: for example, 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 mg/ml BSA, 10 mM-50 mM NaCl, and either 40 mM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 14°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 mg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations are performed at 1 mM total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5'

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phosphate and prevent self-ligation of the vector. Digestions are conducted at pH 8 in approximately 10 mM Tris-HCl, 1 mM EDTA using about 1 unit per mg of BAP at 60°C for one hour or 1 unit or CIP per mg of vector at 37°C for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion and separation of the unwanted fragments.

Example 1
Coincidence Cloning of Blunt/Sticky-End Heteroduplexes

This example describes the use of coincidence cloning to identify common genomic sequences in LAZ342, a human lymphoblastoid cell line, and the somatic cell hybrid HHW661, a hamster-human hybrid cell line containing only a single human chromosome: a translocation of human chromosome region 4p onto hamster chromosome 5. The HHW661 cell line was prepared according to published methods (Wasmuth).

Genomic DNA from the two cell lines was obtained by conventional methods (Maniatis), and both DNAs were cut to completion with MboI, which generates fragments predominantly less than 1 kb in length. With reference to Figure 1, the HHW661 DNA fragments were further blunt-ended with Klenow fragment in the presence of all four nucleotides, so that the final HHW661 fragments are blunt-ended homoduplexes (DNA-I fragments in the figure) and the lymphoblastoid cell fragments are sticky-ended (DNA-II in the figure).

Both mixtures of DNA fragments were mixed in a 1:1 ratio, alkaline denatured at pH 13, and then reannealed by a phenol emulsion reassociation technique

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(F-PERT) (Kohne; Casna). Specifically, the denatured DNA fragments were mixed, and phenol and formamide were added to final volume concentrations of 27 and 8 percent, respectively. A two-phase emulsion was formed by vigorous shaking with a vibratory shaker run at 1/2 to 3/4 maximum speed. Total reannealing time was about 20-24 hours at 22° C. The annealed DNA was recovered by phenol extraction and ethanol precipitation, according to known methods. As seen from Figure 1, the reannealed fragments include original blunt-ended homoduplexes from the DNA-I fragments, DNA-II homoduplexes with opposite MboI sticky ends, and heteroduplexes with opposite MboI (or BamHI) and blunt ends, as indicated. Reassociation of repeat sequences in the fragment mixtures would not be expected to yield clonable ends, since the repeats are likely to hybridize with imperfect copies of themselves.

pUC18 plasmids were treated with BamHI and SmaI restriction endonucleases, to cut the plasmid in its polylinker region, and the small linker fragment was removed by PEG precipitation. The reassociated fragments from above were mixed and ligated with the cut plasmids under standard conditions. Since the MboI ends of the heteroduplex are compatible with the BamHI and SmaI ends of the cut plasmid, respectively, only the heteroduplex fragments are expected to form successful recombinants. The recombinant plasmids are used to transform JM103 host cells, and successful transformants are selected by plating in the presence of isopropylthiogalactoside (IPTG) and 5-bromo-4-chloro-3-methyl-indolyl-beta-D-galactoside (Xgal). Minipreps of the plasmid DNA, designated pUC/HD in Figure 1, revealed detectable inserts in the 200-1,000 bp range in 60% of the clones. Screening 48 colonies with total hamster DNA by a colony filter hybridization technique under conditions that only permit hybridization of repeated sequences (Maniatis, p. 316) showed no positives, and only 1 colony was positive for total human

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DNA under the same conditions, indicating that the method of the invention selects against repeated sequences.

Five inserts were purified from low-melting temperature agarose (Sea Plaque) and labeled using random hexamer priming (Feinberg). The five labeled fragments were used as probes on Southern blots containing hamster DNA, HHW661 DNA, and human DNA. Three of the five probes gave single-copy bands in the HHW661 and human DNA lanes, and no signal in the hamster lanes, indicating that these fragments did in fact arise from the human translocation chromosome, as expected.

Example 2
Coincidence Cloning with Mixed Sticky-End Linkers

Genomic DNA from the lymphoblastoid and HHW661 cell lines above is cut to completion with MboI, as described, yielding predominantly 200-1,000 bp fragments with MboI sticky ends, as illustrated in Figure 2, where again the HHW661 fragments are indicated DNA-I and the lymphoblastoid-cell fragments, as DNA-II.

Synthetic linkers having an MboI sticky end and an internal XhoI site (linker I in the figure) or an internal NotI site (linker II) are prepared by conventional oligonucleotide methods, as described above. The XhoI linker is ligated to the DNA-I fragments, and the fragments are cut to completion with XhoI endonuclease, yielding DNA-I fragments with XhoI sticky ends, as indicated. Similarly, the DNA-II fragments are ligated with the NotI linker, and the resulting fragments are cut to completion with NotI endonuclease, yielding DNA-II fragments with NotI sticky ends as shown. Here it is noted that fragments with relatively rare internal XhoI or NotI sites will not form the desired heteroduplexes (below) and thus will be lost.

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Both mixtures of DNA fragments are mixed in a 1:1 ratio, alkaline denatured at pH 13, then reannealed by the formamid-phenol emulsion reassociation technique (F-PERT) of Example 1. The annealed DNA is recovered by phenol extraction and ethanol precipitation, as above. As seen from Figure 2, the reannealed fragments include the original homoduplexes from the DNA-I and DNA-II fragments, having opposite XhoI and NotI ends, respectively, repeat sequences with different-length ends, and heteroduplexes with opposite XhoI and NotI ends.

A Bluescript® plasmid containing NotI and XhoI sites in the plasmid's polylinker region is cut with XhoI and NotI endonucleases, and the small linker fragment is removed by polyethyleneglycol (PEG) precipitation. The reassociated homoduplex and heteroduplex fragments from above are mixed and ligated with the cut plasmids under standard conditions. As can be appreciated from above, and from Figure 2, only the end-hybridizable heteroduplexes, with their opposite NotI and XhoI sticky ends are compatible with the cut ends, of the plasmid, and therefore only these heteroduplexes are expected to form successful recombinants. Confirmation of successful recombinant plasmids is on JM103 host cells, with plating in the presence of isopropylthiogalactoside (IPTG) and Xgal, as above. Minipreps of the plasmid DNA are used to test plasmids with inserts in the 200-1,000 bp size range.

Non-repeat clones are further purified and labeled as above, for screening genomic fragments from hamster, human and HHW661 cells, to identify those clones which are specific for both human and HHW661 genomic fragments, as determined, for example, by probe binding to Southern blots of the genomic fragments.

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Example 3
Coincidence Cloning with Methylated Heteroduplexes

Genomic DNA from the lymphoblastoid and HHW661 cell lines above is cut to completion with BamHI, yielding fragments predominantly in the 2-10 kilobase size region and having BamHI (B) sticky ends, as seen in Figure 3, where the fragments derived from the HHW661 and lymphoblastoid cell lines are designated DNA-I and DNA-II, respectively. The DNA-I fragments are now treated with AluI methylase, to block internal AluI (A) sites in the fragments, and the DNA-II fragments are similarly treated with HaeIII methylase, to block internal HaeIII (H) sites. Restriction site methylation is indicated by the "*" symbol on both fragment strands in the figure.

Both mixtures of DNA fragments are mixed in a 1:1 ratio, alkaline denatured at pH 13, then reannealed by a phenol emulsion reassociation technique (F-PERT), as above, and the annealed DNA is recovered by phenol extraction and ethanol precipitation, as above. As seen from Figure 3, the reannealed fragments include the original homoduplexes from the DNA-I and DNA-II fragments, having opposite BamHI ends, same-size heteroduplex fragments also having opposite BamHI ends, and unequal-strand homoduplex and heteroduplex fragments (predominantly different-size homologous repeat sequences) having at least one irregular end.

The reannealed fragments are now digested to completion with both AluI and HaeIII under standard digest conditions to cut those fragments at internal, non-methylated EcoRI and MboI sites, respectively. As can be appreciated from Figure 3, AluI and HaeIII digestion of the DNA-I homoduplexes, which have protected AluI sites, produces BamHI/HaeIII (B/H) and HaeIII/HaeIII (H/H) fragments in all fragments which have internal HaeIII sites. Similarly, AluI and HaeIII digestion of the DNA-II

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homoduplexes, which have protected HaeIII sites, produces BamHI/AluI (B/A) and AluI/AluI (A/A) fragments in all fragments which have internal AluI sites. Since the equal-strand heteroduplex fragments are protected at both sites, by methylation of the AluI sites on one strand and the HaeIII sites on the homologous strand, the heteroduplex is not susceptible to digestion and therefore retains its two opposite BamHI sites. It is noted here that the small percentage of homoduplex fragments which do not contain internal AluI or HaeIII sites will also retain their opposite BamHI sticky ends.

The digest fragments from above are now ligated into a pUC18 plasmid which has been linearized by BamHI digestion. As above, the digested fragments are mixed and ligated with the cut plasmids under standard conditions, and the plasmids are selected for successful recombinants, which should contain only the matched heteroduplex fragments. Non-repeat clones are further purified and labeled as above, for screening genomic fragments from hamster, human and HHW661 cells, to identify those clones which are specific for both human and HHW661 genomic fragments, as determined, for example, by probe binding to southern blots of the genomic fragments.

Example 4
Coincidence Cloning with Methylated-Linker Heteroduplexes

Genomic DNA from the lymphoblastoid and HHW661 cell lines above is cut to completion with MboI or BamHI, as above yielding predominantly 200-1,000 bp fragments with MboI sticky ends, or 200-20,000 bp fragments with BamHI sticky ends as illustrated in Figure 4. As in Figures 1-3, the HHW661 fragments are indicated as DNA-I and the lymphoblastoid-cell fragments, as DNA-II.

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Synthetic linkers having an MboI (M) sticky end and internal HaeIII (H) and AluI (A) sites and either an XhoI (X) or a NotI (N) site adjacent the opposite linker end, are prepared by conventional methods, as detailed in the Materials and Methods section above. The nucleotide sequence of the two linkers is shown in Figure 4. The XhoI linkers (linker I) are ligated to the DNA-I fragments, yielding fragments having groups of H/A/X sites at each end region. For purposes of illustration, the fragments illustrated in the figure are also shown as having internal AluI (A) and HaeIII (H) sites, since in fact, many of the genomic fragments will contain such sites. The DNA-I fragments with attached linkers are now treated with AluI methylase, to methylate all AluI sites in the fragments, including those in the fragment end linkers. As indicated by the "*" methylation symbol, both strands of the fragments are so methylated. Enzymatic conditions for ligating linkers to the DNA fragments and for methylating the fragments are conventional.

Similarly, the NotI (linker II) are ligated to the DNA-II fragments, yielding fragments having groups of H/A/N restriction sites at each fragment end. Methylation of these fragments with HaeIII methylase gives the fragments indicated with methylated HaeIII sites in both of the DNA-II sites.

The mixtures of DNA fragments are mixed in a 1:1 ratio, alkaline denatured at Ph 13, then reannealed by a phenol emulsion reassociation technique (F-PERT), as above, and the annealed DNA is recovered by phenol extraction and ethanol precipitation, as above. As seen from Figure 4, the reannealed fragments include the original homoduplexes from the DNA-I and DNA-II fragments, having either H/A/X or H/A/N linkers, respectively, at their opposite ends, repeat sequences with different-length ends, and heteroduplexes with an H/A/X linker sequence at one end and an H/A/N linker sequence at the other end.

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The reannealed fragments are now digested to completion with both AluI and HaeIII endonucleases, under standard digest conditions. With continued reference to Figure 4, digestion of the AluI methylated homoduplexes (the DNA-I homoduplexes) with the combination of endonucleases cleaves the fragments at all HaeIII sites, including the end linker sites, producing fragments whose opposite ends have HaeIII blunt ends. Similarly, digestion of the HaeIII methylated homoduplexes (the DNA-II homoduplexes) with the combination of endonucleases cleaves the fragments at all AluI sites, including the end linker sites, producing fragments whose opposite ends have AluI blunt ends. In the single-copy heteroduplex fragments (formed from same-length strands), all of the AluI and HaeIII sites are methylated on one strand or the other, and so no endonuclease digestion occurs, yielding intact heteroduplex fragments with opposite XhoI and NotI ends. Duplex fragments which are not end-hybridized sequences will be cleaved by the AluI or HaeIII endonucleases only in duplexes where the homologous strands are derived from the same original DNA mixture, thus yielding fragments with irregular ends, or fragments where one or both ends are AluI or HaeIII ends.

The digest fragments from above are now ligated into a Bluescript® vector having NotI and XhoI polylinker sites. Briefly, the vector is digested with the both NotI and XhoI, with removal of the small linker fragment. As above, the digested fragments are mixed and ligated with the cut plasmids under standard conditions, and the plasmids are selected for successful recombinants, which should contain only the matched heteroduplex fragments. Non-repeat clones are further purified and labeled as above, for screening genomic fragments from hamster, human and HHW661 cells, to identify those clones which are specific for both human and HHW661 genomic fragments, as

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determined for example, by probe binding to Southern blots of the genomic fragments.

Example 5
Heteroduplex Selection of Mixed-Strand Biotinylated Fragments: Method 1

Genomic DNA from the lymphoblastoid cell line above is cut to completion with HindIII and EcoRI, substantially as described, yielding predominantly 200-10,000 bp fragments with HindIII and EcoRI sticky ends. These fragments are then hybridized with single-copy, biotinylated HHW661 DNA fragments also produced by complete HindIII and EcoRI digestion, and prepared as described in Parts A and B below.

A. Removing Repetitive Sequences from the HHW661 DNA Fragments

The HindIII/EcoRI digest fragments from the HHW661 cell line are dissolved in 0.12 M phosphate buffer containing 0.2 mM EDTA (PB). Repetitive-sequence DNA is removed by standard hybridization methods which are detailed in the literature (Britten). Briefly, the DNA is raised to about 10°C above the melting temperature (T_m), as determined for example by absorption at OD_{260}. In the buffer used above, the T_m is between about 80°-90°C. The material is then cooled slowly to about 25°C below the T_m, and allowed to anneal to a $C_o t$ value (mole/liter x sec) of about 100, at which the repeat-sequence material is predominantly in reannealed form, and the non-repetitive fraction, in denatured form. This duplex material is separated from single-strand DNA by hydroxyapatite (HAP) chromatography, according to standard procedures (Britten). Briefly, HAP is suspended in 0.15 PB, 2 mM EDTA, and poured into a water-jacketed column maintained at the reannealing temperature. After washing the column

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with several volumes of the reannealing buffer, the DNA material is loaded onto the column and the single-strand material eluted with several volumes of the buffer. This material is combined, and precipitated with cold ethanol, as above.

The precipitated single-strand material is redissolved in annealing buffer, and the entire separation procedure repeated, except that the reannealing is performed at a temperature about 10° below the above T_m value.

B. Biotinylating the Single-Copy HHW661 Fragments

The biotinylated nucleotides used are Bio-11-dUTP (Brigati) which has an 11-atom linker arm separating the biotin and the pyrimidine base, and Bio-19-SS-dUTP (Herman) which has a 19-atom linker containing a disulfide bond. ^{32}P-labeled dNTPs are included when monitoring of the various steps of the method is desired. The labeled nucleotides are incorporated into the double strand fragments by one of the following methods:

1. Nick-Translation

A typical reaction, carried out in 60ml final volume, contains 1 ug DNA in 50 mM Tris-Cl pH 7.5, 10mM MgSO4, 0.1 mM DTT, 100 mM of each of the following nucleotides: dATP, dGTP, and Bio-11-dUTP or Bio-19-SS-dUTP, 5 uCi of [alpha-^{32}P] dCTP (Amersham, specific activity 3,000 Ci/mmole), 30 U DNA polymerase I, and 27 pg/ml DNAse I. The reaction mixture is incubated at 14°C for one hour, stopped by addition of EDTA to 10 mM and heated at 68°C for 5 min. Labeled DNA is recovered by chromatography over Sephadex G50 equilibrated and eluted with 10 mM Tris-Cl, pH 7.5/1 mM EDTA (T.E.). When large amounts of DNA are required, two to three nick-translations are run in parallel and loaded onto one column to obtain a concentrated DNA solution.

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2. Tailing by Terminal Transferase

This procedure is used only after the DNA is first treated to produce 3' protruding ends (Maniatis). The reaction mixture consists of 1 ug DNA in 100 mM potassium cacodylate (pH 7.2), 2 mM $CoCl_2$, 0.2 mM DTT, 100 mM Bio-11-dUTP, 50 mCi [alpha-^{32}P] dCTP, and 20 U terminal transferase, added last. After incubation at 37°C for 45 min, an additional 20 U of enzyme is added and the incubation repeated. The reaction is terminated by EDTA added to 10 mM, the DNA is recovered as described above, precipitated with ethanol, washed with 70% ethanol and resuspended in 50 ul buffer.

3. Labeling by T4 DNA Polymerase Replacement Reaction

The reaction contains 1 ug of DNA in 33 mM Tris-OAc (pH 7.9), 66 mM NaOAc, 10 mM MgOAc, 0.5 mM DTT, 0.1 mg/ml BSA, and 0.5 U T4 DNA polymerase. After incubation at 37°C for 7 minutes, dATP, dGTP, and Bio-11-dUTP are added to a final concentration of 150 mM, dCTP is added to 10 mM, 50 mCi of [alpha-^{32}P] dCTP (3000 Ci/mmole), and TrisOAc, NaOAc, MgOAc, BSA, and DTT are added to maintain previous concentrations. This reaction is incubated at 37°C for 30 min, then dCTP is added to a concentration of 150 mM, and the reaction incubated for an extra 60 min at 37°C. The reaction is stopped by addition of EDTA to 10 mM, heated at 65°C for 10 min, chromatographed and processed as described before.

4. Labeling by Photobiotinylation

This is carried out by standard procedures, as outlined in the protocol supplied by the manufacturer (Clontech, Palo Alto, CA).

C. Selection of Mixed-Strand Heteroduplex Fragments

With continued reference to Figure 5, the HindIII/EcoRI fragments from the human lymphoblastoid line

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are cloned into the HindIII and EcoRI sites of M13, and the plasmid is grown under conditions which produce M13 supernatant phage containing the inserts in single-strand form. The phage material is harvested and mixed with the biotinylated single-copy HHW661 fragments prepared as in Parts A and B. The fragments are denatured and reannealed using the F-PERT method referenced above.

The reannealed material is passed over an avidin column, for binding of biotinylated DNA to the column material. A 1 ml silanized syringe plugged with silanized glass wool is packed with 0.3 ml streptavidin-agarose and washed with 0.15 PB, 2 mM EDTA. The hybridization mixture from above is loaded onto the column which is then washed with several volumes of the same buffer, to remove non-hybridized cDNA.

The material bound to the column is alkaline denatured at pH 13, and the released (non-biotinylated) DNA strands are eluted with the same high pH medium. The non-biotinylated strand material which elutes is carried in the single-stranded phage. This material, which constitutes human lymphoblastoid DNA sequences which are homologous to single-copy sequences from the HHW661 sequences, is transfected into the JM103 host, and grown in either single-strand or double-strand form.

Example 6
Heteroduplex Selection of Mixed-Strand Biotinylated Fragments: Method 2

Genomic DNA from the lymphoblastoid cell line and HHW661 line are each digested to completion with MboI, and the HHW661 MboI fragments are biotinylated according to Example 5A. The biotinylation is preferably carried out by a method, such as nick translation or terminal tailing with T4 DNA polymerase, which does not alter the sticky end sequences of the fragments. Rather than

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initially removing repeat-sequences from the mixture of the two fragments, the fragments are hybridized under F-PERT conditions, as above, yielding homoduplexes and heteroduplexes which contain both end-hybridized and non-end-hybridized fragments, as illustrated in Figure 6.

The reannealed material is fractionated on a streptavidin column as above, and non-biotinylated bound DNA strands are released by alkaline denaturation as above. The released single-strand species contain both single-copy and repeat-sequence strands which are in common between the two fragment mixtures.

The single-strand eluate fraction from above is ethanol precipitated and reannealed using the F-PERT procedure, resulting in two populations of double-strand fragments, as seen in Figure 5. These include fragments formed by reannealing of end-hybridizable strands, giving duplex fragments in which the original MboI ends are restored, and fragments formed by reannealing of non-end-hybridizable strands, which do not have defined sticky ends.

The reannealed fragments from above are mixed with pUC18 plasmid which has been linearized by digesting with BamHI, and the MboI-ended fragments are ligated into the cut plasmid according to standard procedures. Successful recombinants are selected as in Example 1, and the colonies are screened with repeat-sequence probes, also as above, to identify single-copy clones.

Example 7
Selection of Heteroduplexes with Mixed-Density Strands

Genomic DNA from the HHW661 cells is cut to completion with MboI, and the digest fragments are density labeled with N^{15} nucleotides which are incorporated into the two fragment strands by nick translation, or terminal tailing with T4 polymerase, according to methods described

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above for biotinylating fragments. DNA from the lymphoblastoid cell line above is cut to completion with MboI for hybridization with the labeled HHW661 fragments.

The density-labeled MboI fragments (wavy-line duplex fragments in Figure 7) are mixed with the unlabeled lymphoblastoid-cell fragments (straight-line duplex fragments in the figure), denatured, and reannealed using the F-PERT method, as described above. As shown in the figure, the annealing/reannealing process yields homoduplexes labeled at neither or both strands, and heteroduplexes labeled in one strand only. Among both classes of fragments, homoduplex and heteroduplex, there are matched-strand fragments formed predominantly from single-copy, same-size strands, and unmatched-strand fragments formed predominantly from repeat sequences with different sizes. As above, the matched-strand fragments will have MboI sticky ends, whereas the unmatched-strand fragments will not.

The reannealed mixture is fractionated by equilibrium centrifugation in a CsCl gradient, according to classical techniques (Meselson). At equilibrium, the fragments will have partitioned into three gradient bands, as indicated at the right in Figure 7. These three bands, progressing toward greater density, are: unlabeled homoduplexes; heteroduplexes (containing a single labeled strand), and labeled homoduplexes. These bands are identified by UV absorption, and the heteroduplex band is removed by aspiration.

End-hybridized heteroduplex fragments are selected by cloning into a vector BamHI site, as in Example 5, and the cloned inserts may be further screened to remove repeat sequences.

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Example 8

Heteroduplex selection

Genomic DNA from the HHW661 and human lymphoblastoid cell lines are digested to completion with EcoRI and HindIII. The digest fragments from the HHW661 line (DNA-I fragments in Figure 8) are cloned into the EcoRI/HindIII site of vector M13/mp19 which carries an EcoI to HindIII orientation in its polylinker, to place the fragments in a "5'-3'" orientation in the double-strand vector. Similarly, the digest fragments from the lymphoblastoid line (DNA-II fragments in Figure 8) are cloned into the HindIII/EcoRI site of vector M13/mp19 which carries a HindIII to EcoRI orientation in its polylinker, to place the fragments in a "3'-5'" orientation in the vector.

The two vectors with their two inserts are grown under conditions of phage production, and the phage harvested from the colony supernatant by conventional methods. The phage from the M13/mp19 vector, which produce the "plus" strand of the fragment are mixed with the phage of the M13/mp18 vector, which produce the "minus" strand of the fragment insert. The two phage populations are rapidly annealed using the F-PERT method described above. The duplex material, representing homologous strands from the different DNA mixtures, is separated from single-strand DNA by hydroxyapatite (HAP) chromatography, according to standard procedures (Britten). Briefly, HAP is suspended in 0.15 PB, 2 mM EDTA, and poured into a water-jacketed column maintained at the reannealing temperature. After washing the column with several volumes of the reannealing buffer, the DNA material is loaded onto the column and the single-strand material is eluted with several volumes of the buffer. The duplex material is eluted at elevated temperature with buffer.

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As seen in Figure 8, the heterologous duplex fragments include end-hybridized inserts in which the vector polylinker sites EcoRI (R) and HindIII (H) on the opposite sides of the insert are aligned and homologous, and non-end-hybridized fragments in which at least one of the polylinker ends is unmatched. The heterologous duplex material is now digested to completion with EcoRI and HindIII to release end-hybridized heteroduplex inserts with opposite EcoRI and HindIII ends, and these fragments are cloned in the EcoRI/HindIII site of a pUC18 vector, as above. Alternatively, the relatively small EcoRI/HindIII fragments can be separated by gel electrophoresis, or by hybridization of the fragments with opposite-strand, biotinylated M13 vector, and removal by streptavidin affinity chromatography, as in Example 5.

Example 9

Isolation of Unique Genomic Restriction Fragment

This section describes the isolation and cloning of sequences from a unique SalI fragment from the human genome. The method involves first performing a partial digestion of the genome with SalI. The partial digest fragments, which have size ranges from a few to up to several thousand kilobases, are fractionated by pulsed-field gel electrophoresis, and the gel is probed with a radiolabeled, selected-sequence probe. Two of the gel regions which are positive for hybridization to the probe are eluted, digested completely with MboI, and the coincidence cloning method of the invention is used to identify and isolate sequences from the unique SalI fragment in each fragment mixture which binds to the selected probe. Details of the method are as follows:

A. Partial SalI Digestion

Peripheral blood lymphocytes (PBL) are pelleted by low speed centrifugation, and washed two times with 10

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ml of phosphate-buffered saline (PBS). The cells are suspended to a final concentration of about 1 x 10^7 cells/ml and a portion of the suspension is mixed with an equal volume of 1% low-gelling temperature agarose. The agarose mixture is cooled to 45-50° C and immediately pipetted into a mold that makes 100 ul blocks, each about 2mm x 5 mm x 10 mm. The blocks are solidified by contacting the mold with ice.

The cells are disrupted in the agarose blocks by incubating the blocks for 2 days at 50° C with gentle shaking in ESP buffer (0.5 M EDTA, pH 9.0, 1% sodium dodecyl sulfate (SDS), and 1 mg/ml proteinase K). After incubation the samples are stored at 4° C in ESP.

Prior to restriction endonuclease digestion, the blocks are treated with PMSF to inactivate proteases in the block. This is done by treating each block twice with 1 ml of 1mM PMSF in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4), with slow rotation at room temperature for 2 hours. This is followed by three 1 ml washes with Tris-HCl buffer alone, for two hours each.

Partial digestions are carried out in 1.5 ml microfuge tubes containing 100 ug/ml bovine serum albumin and SalI in 10 mM Tris-HCl buffer, pH 7.4, to a final volume of 250 ul. The agarose blocks are added to the tubes before the addition of SalI. The final concentration of SalI is either 2, 5, or 10 units/ug DNA in the block. For blocks prepared as above and containing about 10 ug DNA, SalI is added to the tubes to a final amount of 10, 50, or 100 units. The tubes are incubated at 37° C for increasing time periods ranging from 30 minutes to 12 hours. To terminate the digestion, the buffer in a tube is carefully aspirated, and replaced with 1 ml of ES buffer (ESP without proteinase K), and the block is incubated in this buffer for 1 hour at 4°C. The buffer is then removed, replaced with 250 ul of ESP, and incubated an additional 2 hours at 50°. After aspirating the

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buffer, the block may be placed directly in an agar slab (below) for pulsed field gel electrophoresis (PFGE), or stored at 4° C until use.

Optimal partial digest conditions are determined by running each of the blocks from above on PFGE, and determining the optional incubation period and SalI concentration which give the desired size distribution of partial digest fragments. As seen in Figure 9, under optimal conditions, the genomic fragments will contain between zero to 3 or more internal SalI (S) sites. For purposes of illustration, the SalI fragment of interest in the figure is the S_3/S_4 fragment, which is also contained in the S_2/S_4 and S_1/S_4 fragments shown in the figure. In particular, the S_3/S_4 fragment is a relatively large genomic fragment which contains (a) a single-copy gene sequence which is homologous to the labeled probe, and (b) a gene region of interest. In general, the fragment of interest is too large to clone as a single piece, and the probe-sequence region may be separated from the gene region of interest by typically more than about 50 and up to 1,000 kilobases.

B. Size Fractionation by PFGE

The SalI partial digest fragments from above are fractionated by PFGE, substantially according to published methods (Smith; Schwartz). Briefly, a gel suspension containing 1.0% agarose, and TBE buffer (10 mM Tris/Borate buffer, pH 7.4 containing 0.1 mM EDTA) is poured into a 20 cm^2 mold to a depth of about 12 mm. After gel hardening, slots corresponding in size to the gel blocks are cut along one edge of the gel, and the gel blocks, typically one every 2 cm, are placed in the slots. The slab is placed in a horizontal gel box containing electrodes on all four sides, at an angle of 45° with respect to the sides of the box, i.e., such that the diagonals of the box

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are normal to the sides of the slab. The arrangement of electrodes described generally in Carle is used.

Electrophoresis is carried out with continuous circulation of TBE buffer, with cooling of the circulated buffer at 15°C, at pulse times of about 60 seconds at 200 volts. The electrophoretic run is terminated when the marker bands have migrated to near the bottom of the gel, as indicated by ethidium staining. Typical electrophoresis times are between about 24 and 36 hours. The gel is cut in half, providing one gel for use in Southern blotting, and a second gel for use in obtaining intact duplex SalI fragments. These two gels are referred to below as "probe" and "recovery" gels, respectively.

C. Identifying Gel Bands

The probe and recovery gels from above are stained by incubation in 1 ug/ml ethidium bromide for 10 minutes with gentle agitation on a platform shaker. The gels are exposed briefly to a weak 360nm UV source during which time photographs of the gel are taken. The two gels are matched for corresponding stained regions, i.e., each stained band in the recovery gel is matched with a corresponding band in the probe gel.

The probe gel is protected from light during subsequent manipulations prior to and during Southern blotting (Smith). Exposure to 254 nm UV light is for one minute. Denaturation of gel DNA material is carried out for one hour in 0.5 NaOH, 0.5M NaCl, and neutralization is carried out for one hour in 1.5M Tris-Cl pH 7.5 with gentle agitation. The gel is blotted to nitrocellulose by ascending transfer overnight with a conventional sodium citrate buffer (Maniatis). The filter is baked for two hours in vacuo at 80°C, and stored in a tight container. Using the labeled probe of interest, a Southern blot of the gel fragments is prepared, according to standard methods (Maniatis). From the blot, two probe-binding gel

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band regions, such as the regions identified as containing fragments S_1/S_4 and S_3/S_4 in Figure 9, are identified. From the positions of these two gel regions, the corresponding regions in the recovery gel are removed for recovery of the fragments in each region. The fragment material is eluted from the gels by electroelution according to standard procedures, and the eluted DNA fragments are ethanol precipitated.

D. Cloning Single-Copy Sequences from the Selected Digest Fragment

The two SalI fragment mixtures obtained from the two gel regions above are each digested to completion with MboI, and the resulting fragments in one of the mixtures is further treated with Klenow fragment in the presence of all four nucleotides, as in Example 1, to fill in the sticky MboI ends. The two fragments mixtures are then mixed, denatured at pH 13, and reannealed by the F-PERT method, as in Example 1, to generate end-hybridized heteroduplexes which have opposite blunt and MboI sticky ends. The hybridization fragments are then cloned into the BamHI/SmaI site of pUC18, as in Example 1, and successful recombinants are identified and screened, both to remove repeat-sequence clones, and to identify clones which hybridize to the labeled probe used above to identify SalI fragments of interest on the PFGE gel. The methods of Examples 2-8 could also be applied.

Example 10
Removing Repeated Sequences from Genomic DNA Fragments

Genomic DNA is obtained from human PBLs as in Example 1, and this material is digested to completion with MboI as in Example 1. The digest material is divided into two equal portions, and one portion is further treated with Klenow fragment in the presence of all four

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nucleotides, as in Example 1, to fill in the sticky MboI ends. The two fragment mixtures are then mixed, denatured at pH 13, and reannealed by the F-PERT method, as in Example 1, to generate end-hybridized heteroduplex sequences from the two mixtures which have opposite blunt and MboI sticky ends. The hybridization products are then cloned into the BamHI/SmaI site of pUC18, as in Example 1. Successful recombinants can further be screened with repeat-sequence probes to remove remaining repeat-sequence clones in the library.

While the invention has been described with reference to particular methods of coincidence cloning, and applications of the method to specific problems in genetic mapping and engineering, it will be apparent to those skilled in the art that various alternative methods and further applications may be developed within the scope of the invention.

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IT IS CLAIMED:

1. A method of obtaining, from a first mixture of DNA duplex fragments derived from a first source, those fragments which are homologous to and end-hybridizable with the duplex DNA fragments in a second mixture of DNA fragments derived from another source, where each duplex is defined as having paired strands, said method comprising

preparing the fragments from at least one of the mixtures so that when a fragment strand from the one mixture is hybridized with a homologous, end-hybridizable strand from the other mixture, the resulting end-hybridized fragment has properties which allow its isolation from homoduplex fragments produced by hybridization between opposite strands of the fragments in the first or second mixture only, and from heteroduplex fragments which are not end-hybridized,

reacting opposite strands from the fragments of the first and second mixtures in a reaction mix under hybridization conditions which yield heteroduplex fragments, and

isolating the end-hybridized heteroduplex fragments from other nucleic acid species contained in the reaction mix.

2. The method of claim 1, wherein said isolating includes introducing the fragments produced by said reacting into a cloning vector which selectively incorporates those end-hybridized heteroduplex fragments.

3. The method of claim 2, wherein the fragments in the two mixtures are generated under conditions which yield a pair of ligatable ends in the end-hybridized heteroduplex fragments which is different from the pair of

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ligatable ends in either the first-mixture or second-mixture homoduplexes.

4. The method of claim 3, wherein the duplex fragments in the first mixture are prepared by (a) cutting duplex DNA with a selected restriction endonuclease which produces fragments with sticky ends, and (b) blunt ending the sticky fragment ends, the fragments in the second mixture are prepared by cutting duplex DNA with a selected restriction endonuclease which also produces fragments with sticky ends, and the heteroduplex fragments have opposite blunt and sticky ends, and the homoduplexes have either opposite blunt or opposite sticky ends.

5. The method of claim 2, wherein said preparing includes attaching to the fragments in the first and second fragment mixtures, end linkers which can be manipulated to yield one ligatable end A at the opposite ends of the first-mixture fragments, and a second ligatable end B at the opposite ends of the second-fragment mixture, and the end-hybridized heteroduplex fragments have A and B ligatable ends at their opposite fragment ends.

6. The method of claim 5, wherein the linkers attached to the first- and second-mixture fragments have internal A and B sequences, respectively, and said preparing further includes cutting each of the first- and second-mixture fragments with an endonuclease which is specific for the A and B sequence, respectively.

7. The method of claim 2, wherein said preparing includes attaching to the first-mixture fragments, an end linker having restriction site sequences A, B, and C, where A and B are internal to C when the linker is attached to the fragments; attaching to the second-mixture

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fragments, an end linker having restriction site sequences A, B, and D, where A and B are internal to D when the linker is attached to the fragments, and C and D are different sequences; treating the first-mixture fragments with a methylase specific for sequence A in the fragments, and treating the second-mixture fragments with a methylase specific for the B sequence in the fragments; and said isolating includes digesting the duplex fragments produced by said reacting with endonucleases which cut the fragments at non-methylated A and B sequences, and cloning the fragments into a cloning vector which incorporates selectively fragments with opposite-end C and D ligatable ends.

8. The method of claim 1, wherein said preparing includes incorporating into the two strands of the fragments in one of the mixtures, a label which allows physical separation of heteroduplex fragments containing one labeled strand from those in which either both or neither fragment strands contain such label.

9. The method of claim 8, wherein the label is an epitopic molecule, and said isolating includes contacting the homoduplex and heteroduplex fragments produced by said reacting with an affinity support material containing a binding molecule capable of binding specifically and with high affinity to the epitopic molecule, to bind fragments in which either one or both strands contain the epitopic label, and treating the support material and attached fragments to denature the fragments and release strands which do not contain the epitopic label, where the pairs of epitopic molecule/binding molecule are selected from the group consisting of biotin/avidin, biotin/streptavidin, carbohydrate/lectin, and antigen/antibody pairs.

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10. The method of claim 9, wherein one of the two fragment mixtures has been prepared to remove repeat-sequence fragments, and the fragment mixture which is not labeled is cloned into a single-strand cloning vector, wherein the unlabeled fragment strands released from the support material can be used to transfect a suitable host, for growth in either single-strand or double-strand form.

11. The method of claim 9, wherein said isolating includes reannealing the unlabeled fragment strands released from the support material, to produce homologous fragments derived from the unlabeled fragment mixture and homologous to sequences in the first mixture, and introducing said reannealed homologous fragments into a cloning vector which selectively incorporates those duplex fragments having ligatable ends.

12. The method of claim 8, wherein said label is an isotopically labeled nucleotide which increases the buoyant density of duplex fragments containing one or both labeled strands, and said isolating includes separating heteroduplex from homoduplex fragments by density centrifugation.

13. The method of claim 12, wherein said isolating further includes introducing heteroduplex fragments produced by said reacting, and having ligatable ends, into a cloning vector.

14. The method of claim 1, wherein said preparing includes cloning the DNA fragments of the first and second mixtures into cloning vectors which can be grown under conditions which yield single strand vectors containing only the sense strands from the first-mixture fragments, and only the anti-sense strands from the second-mixture fragments, said reacting produces

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heteroduplex fragments only, and said isolating includes separating duplex from non-duplex DNA species.

15. The method of claim 14, wherein the first- and second-mixture fragments are cloned in opposite orientations into a single-strand cloning vector, yielding vector sequences A and B on either side of the inserted fragments, and said isolating further includes digesting the heteroduplex fragments with endonucleases with cut at or adjacent said A and B sequences, and cloning the heteroduplex fragments released by said digesting into a vector.

16. The method of claim 1, for use in cloning one or more regions of a DNA restriction fragment contained in a mixture of restriction fragments generated from a DNA source and containing a region which is homologous to a selected DNA probe, comprising

generating the restriction fragments by endonuclease digestion of the DNA source,

fractionating the resulting DNA fragments into several subfractions,

identifying two different subfractions which each bind to a selected probe which is homologous to a region in the restriction fragment of interest, and

applying the method of claim 1 to the two subfractions, to obtain those DNA sequences in the first subfraction which are homologous to sequences in the second subfraction.

17. The method of claim 16, wherein said generating includes partially digesting the source DNA with a rare-cutter endonuclease, said fractionating is performed by pulsed-field gel electrophoresis, said obtaining includes identifying two gel band regions which bind to the probe of interest, and eluting fragments from

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these two regions, and said applying includes digesting the fragments in the two subfractions with one or more restriction endonucleases which reduce the average sizes of the fragments in the mixture to less than about 20 kilobases.

18. The method of claim 1, for use in cloning conserved gene sequences from two different species, comprising

isolating the genomic DNA from the two different species,

digesting the genomic DNA from the two species with one or more selected endonucleases, to produce first and second mixtures of genomic DNA fragments from the two different species, and

applying the method of claim 1 to the two mixtures of DNA fragments, to obtain those DNA sequences in the first mixture which are homologous to sequences in the second mixture.

19. The method of claim 1, for use in enriching a DNA fragment mixture with single-copy sequences, comprising

dividing the mixture into two portions, and

applying the method of claim 1 to the two portions of DNA fragments.

20. The method of claim 1, for use in obtaining cloned sequences from a selected chromosome or chromosome region, comprising

providing a first hybrid cell line which contains such chromosome or chromosome region,

providing a second hybrid cell line which also contains such chromosome or chromosome region, where the two cell lines do not have any other common-species chromosomes,

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obtaining the genomic DNA from the first and second cell lines, and digesting the DNA with one or more restriction endonucleases, to produce first and second mixtures of DNA fragments, respectively, and

applying the method of claim 1 to the two mixtures of DNA fragments, to obtain those DNA sequences in the first mixture which are homologous to sequences in the second mixture.

21. A library of cloned DNA sequences produced by treating two DNA fragment mixtures according to the method of claim 2.

22. The library of claim 21, wherein one of the strands in each heteroduplex fragment contains a nucleotide label not present in the other homologous strand.

23. The composition of claim 22, wherein the label is selected from the group consisting of biotinylated, density-labeled, and methylated nucleotides.

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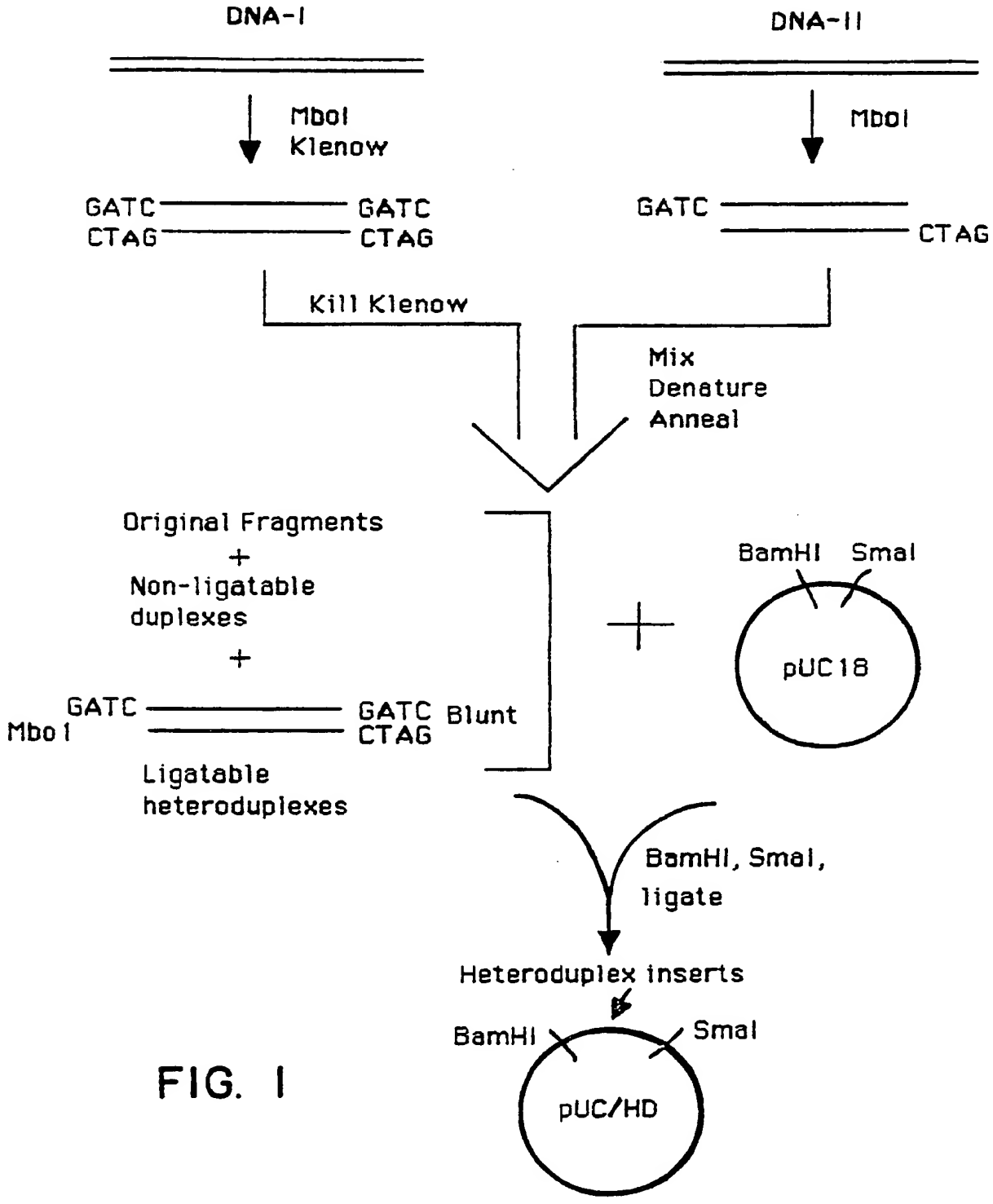

FIG. 1

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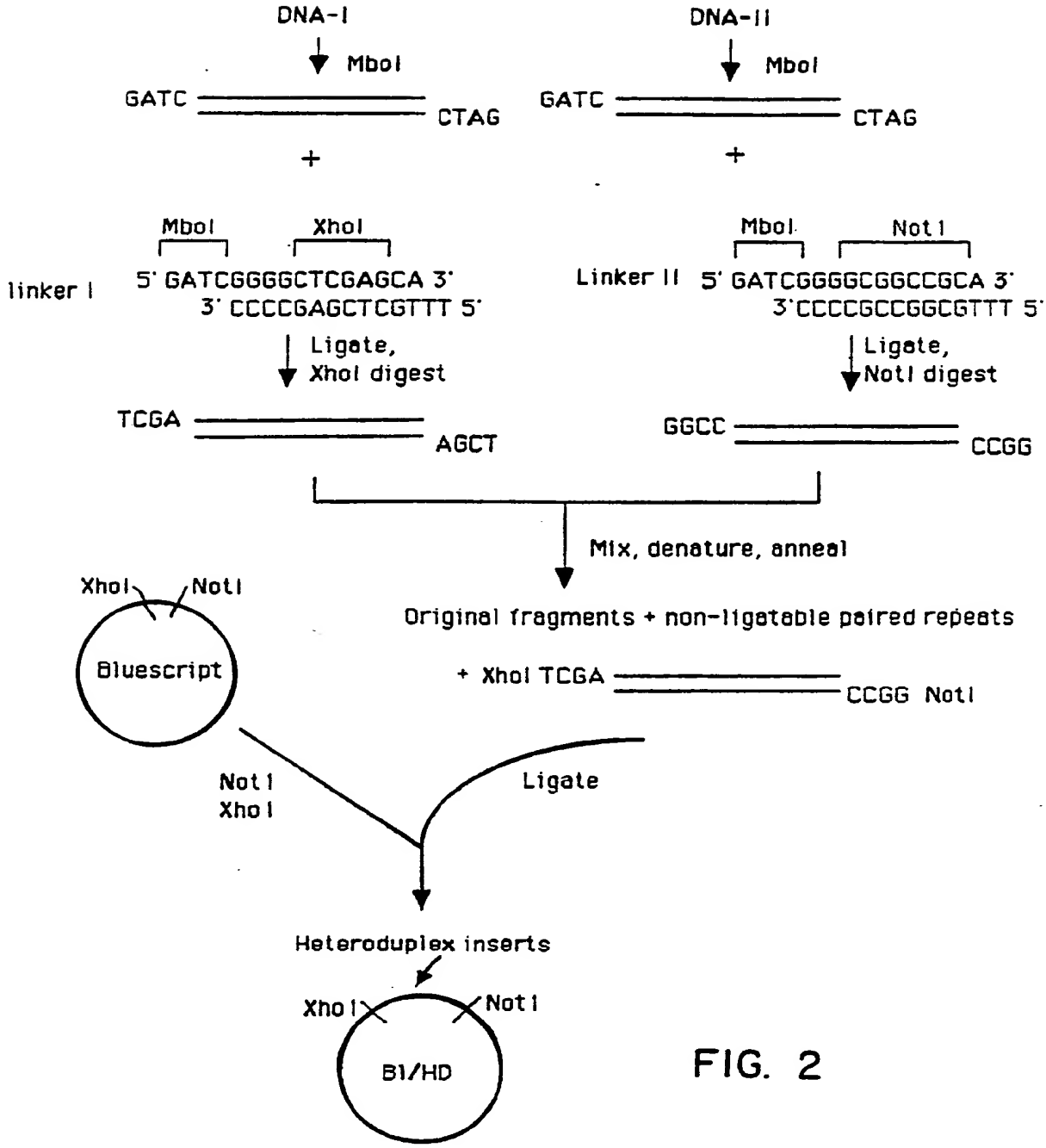

FIG. 2

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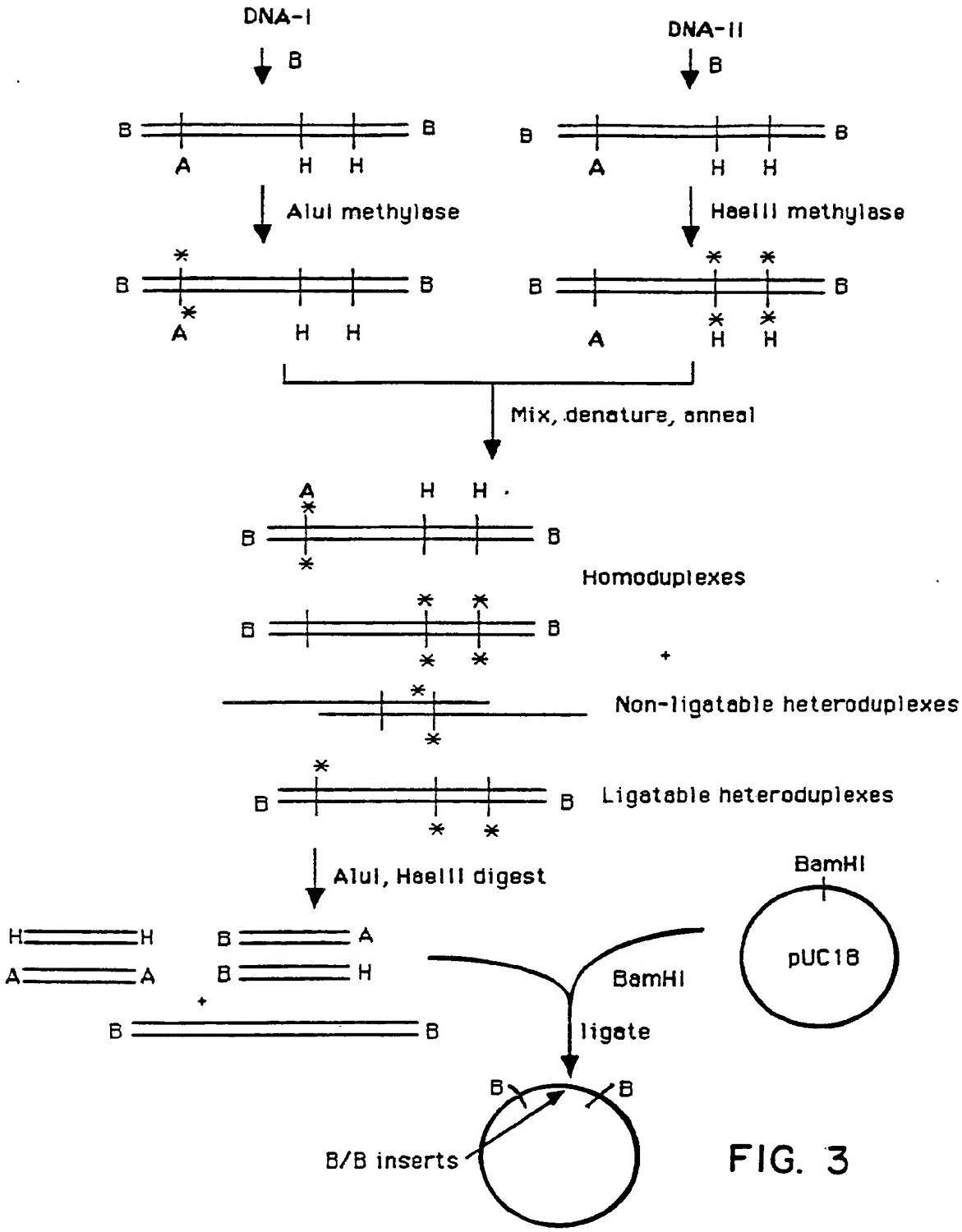

FIG. 3

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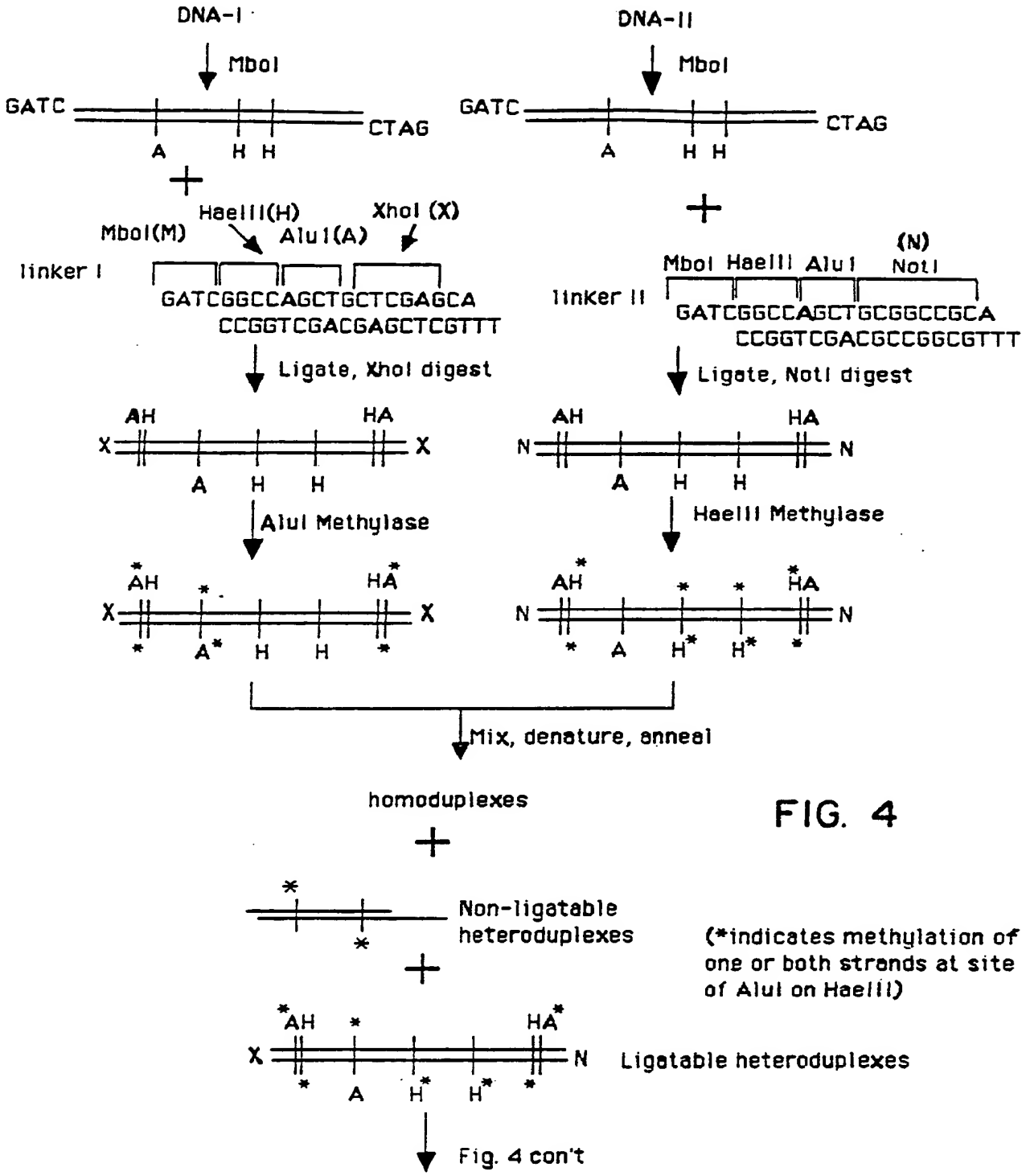

FIG. 4

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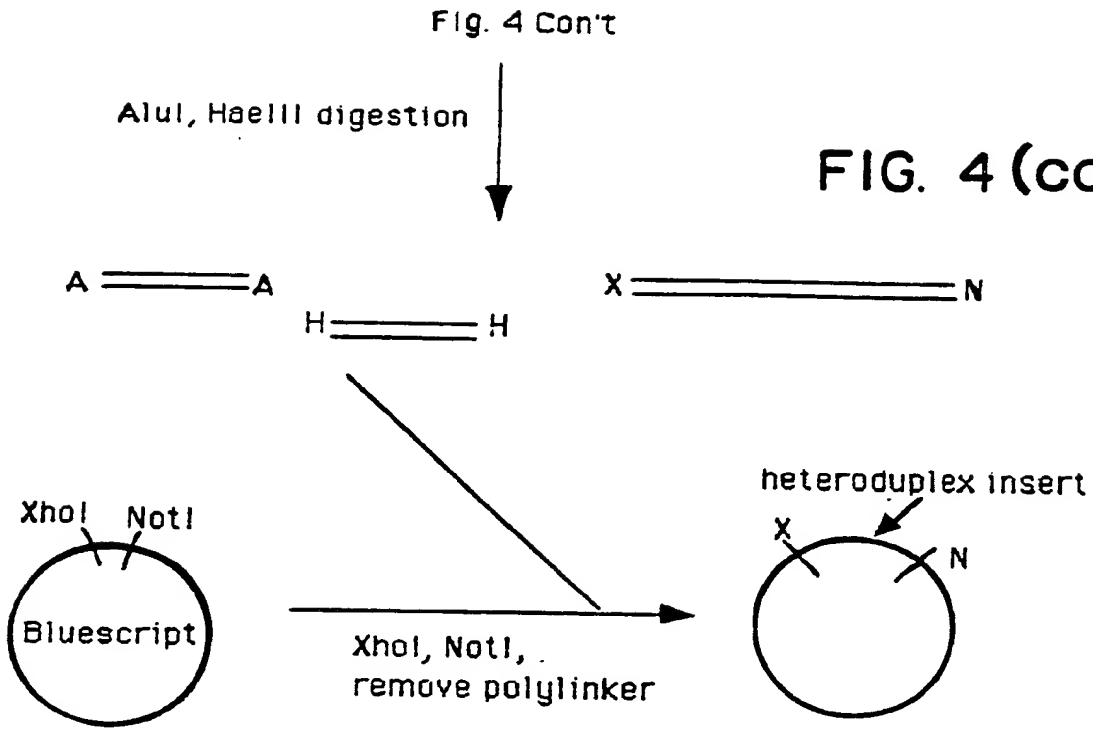

FIG. 4 (con't.)

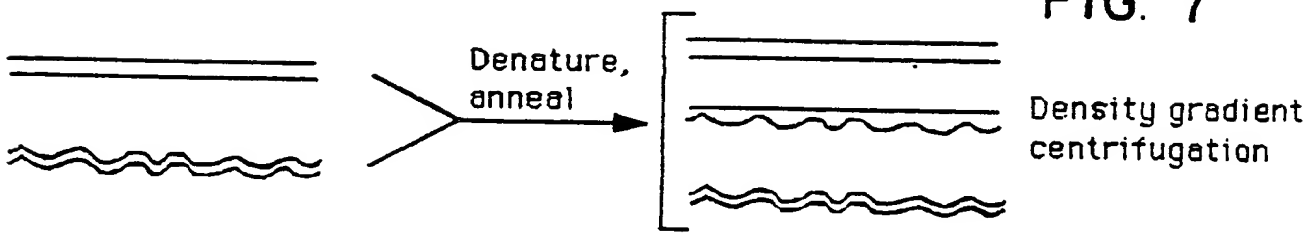

FIG. 7

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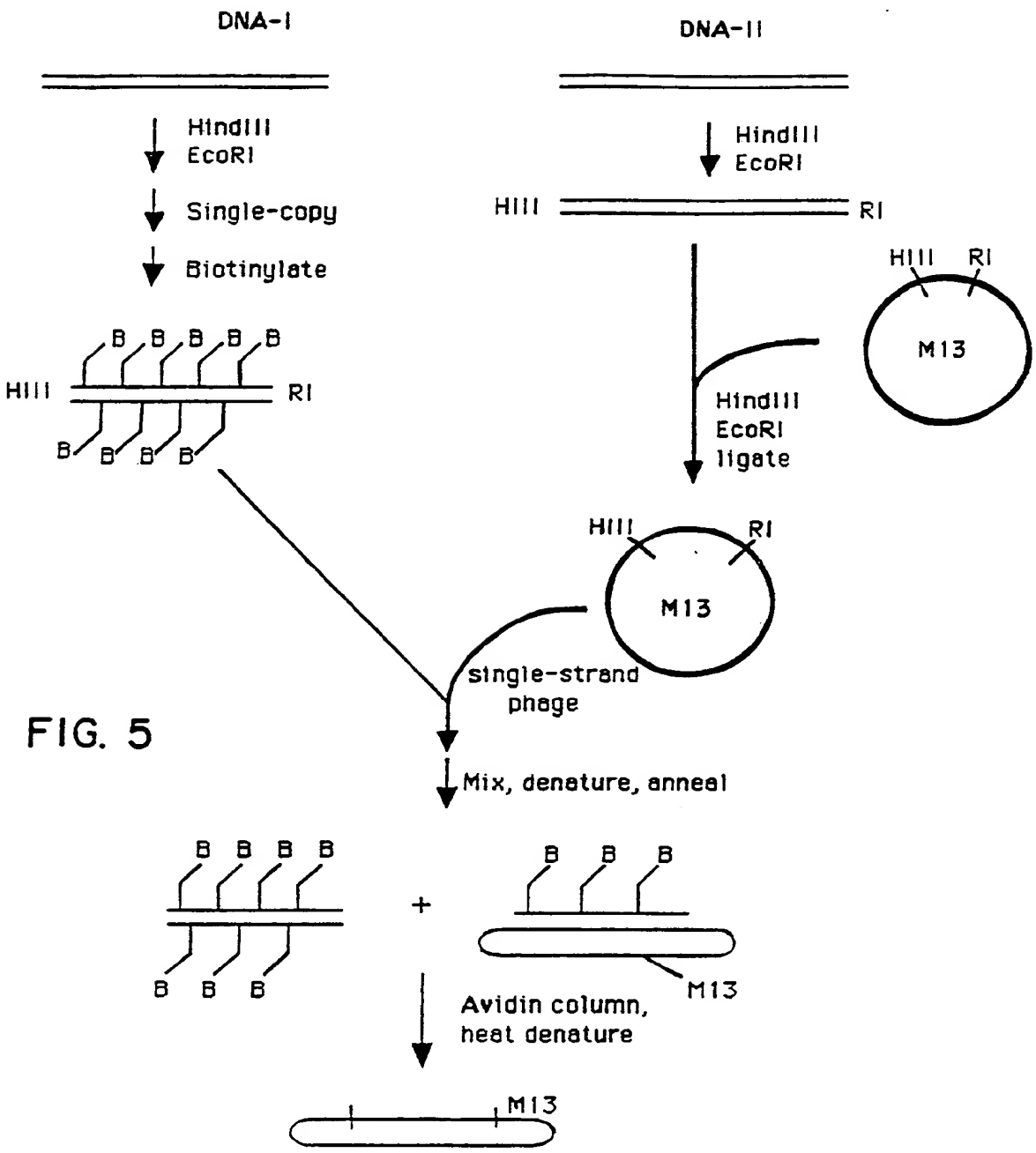

FIG. 5

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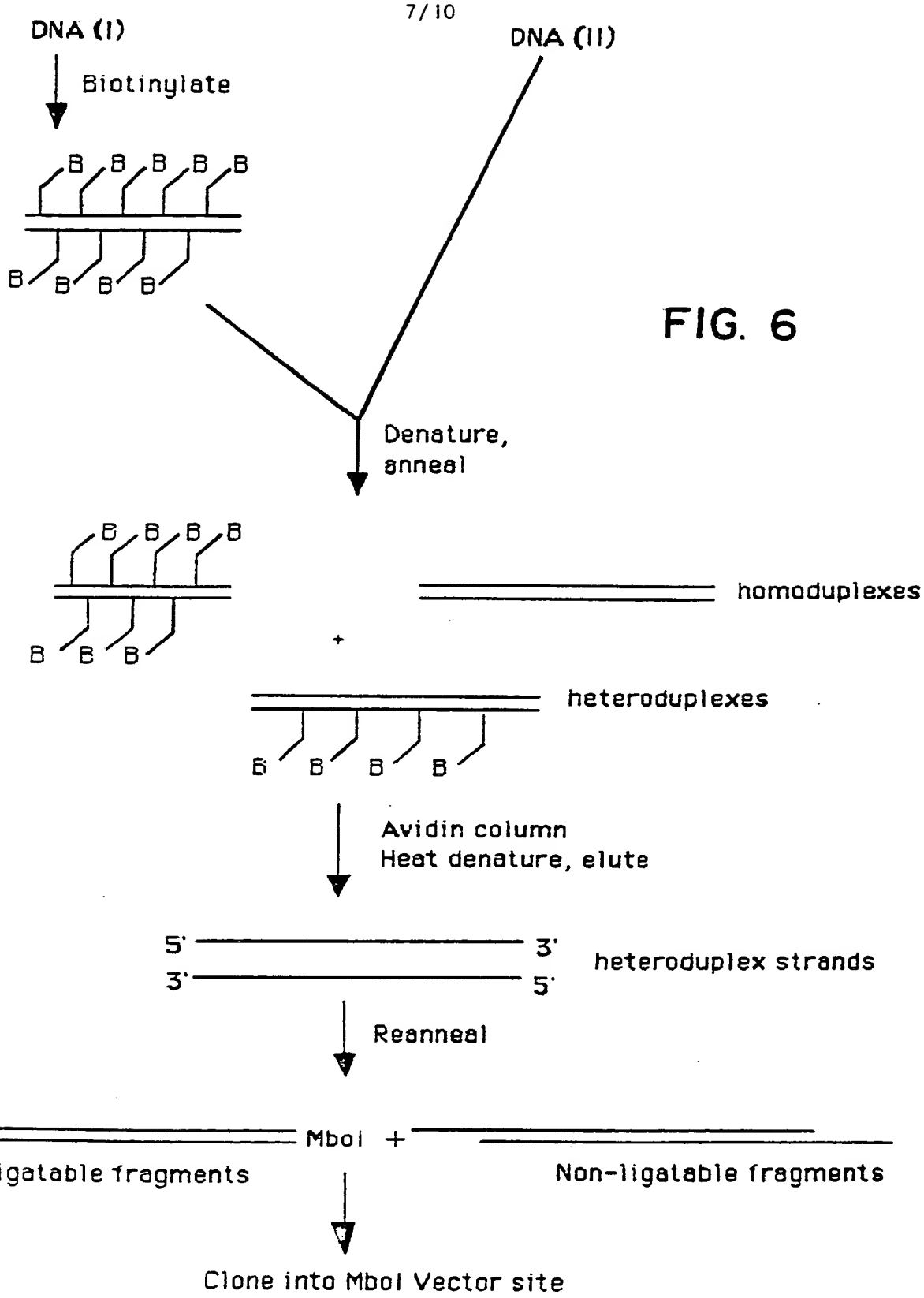

FIG. 6

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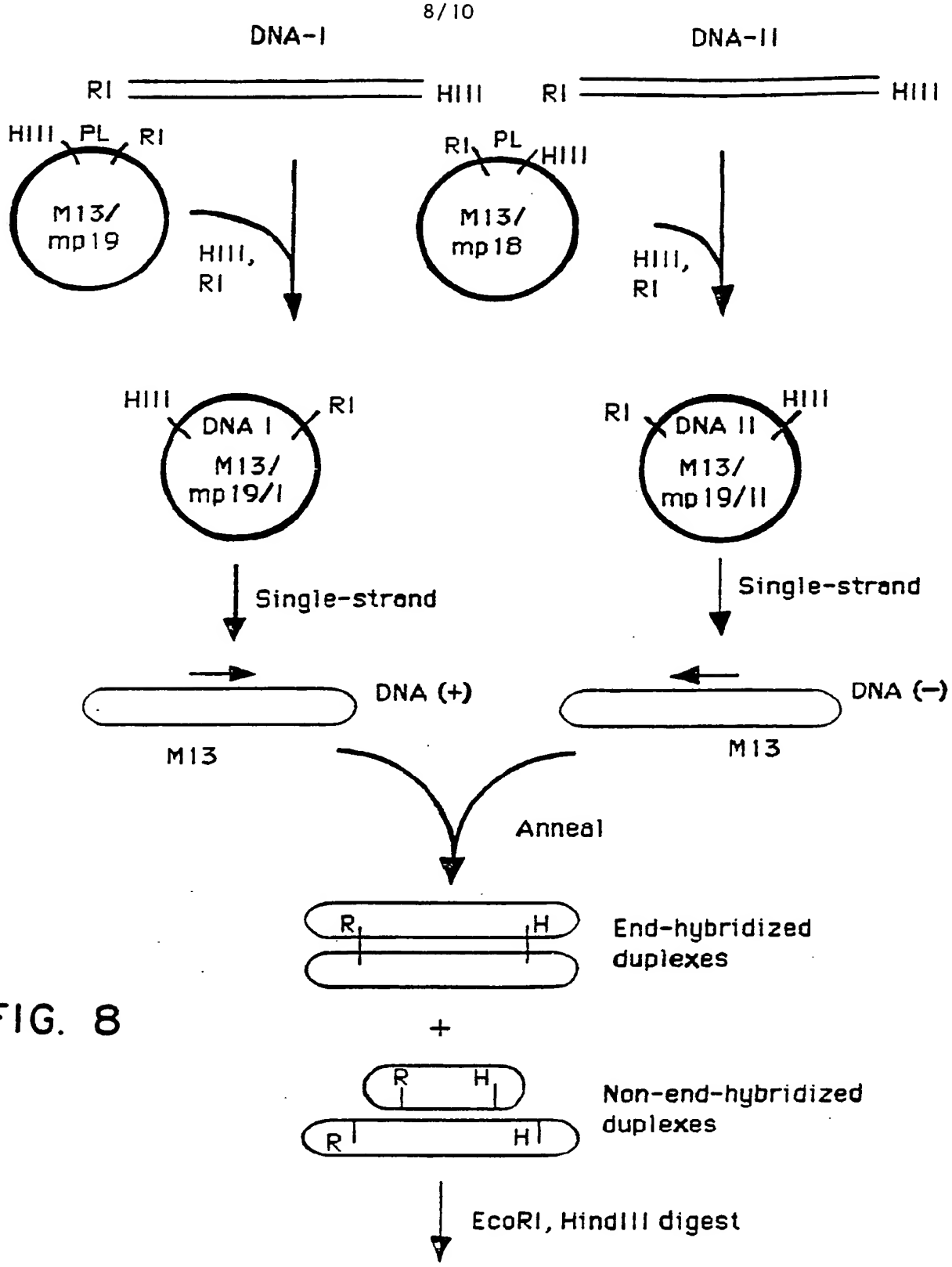

FIG. 8

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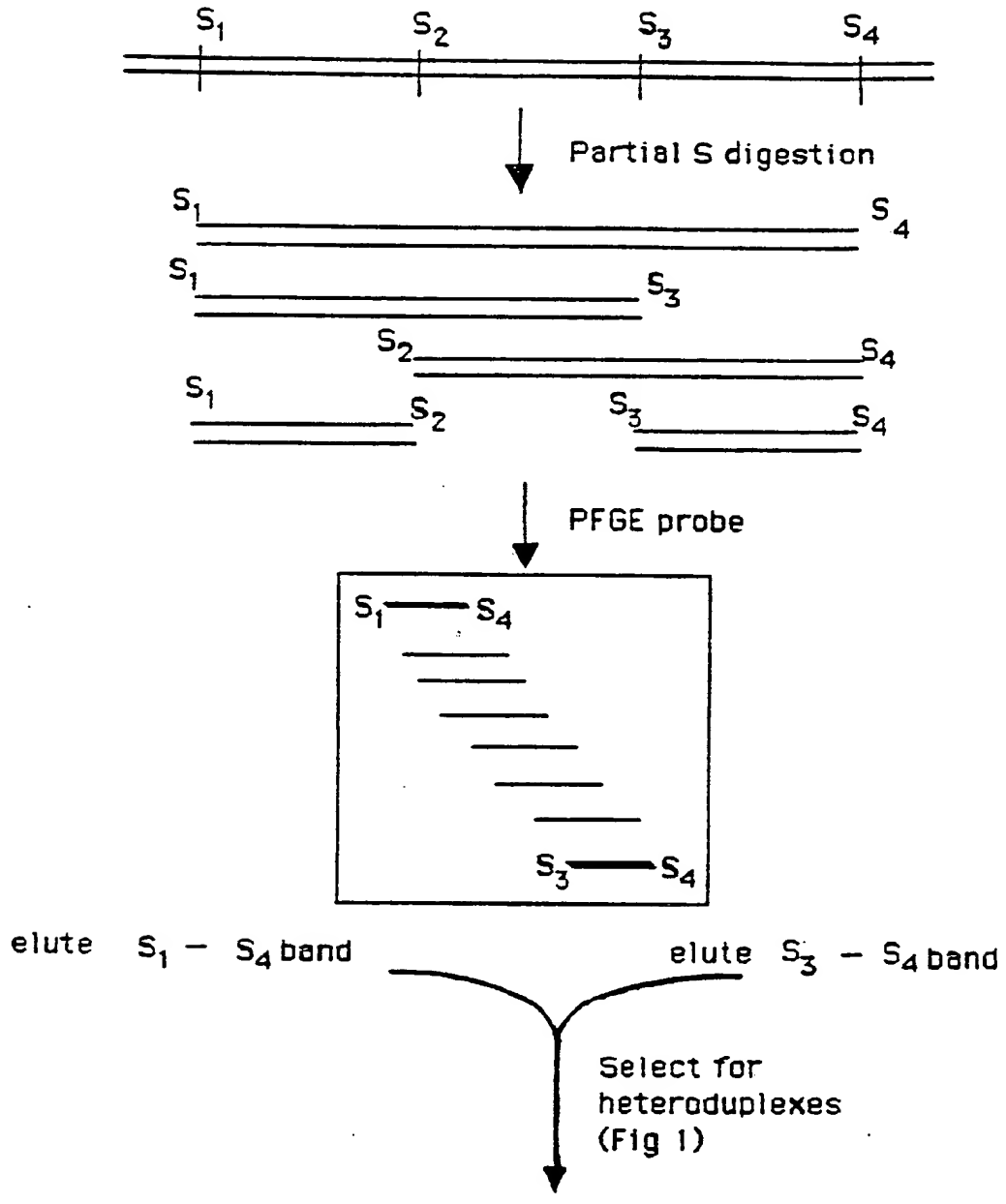

FIG. 9

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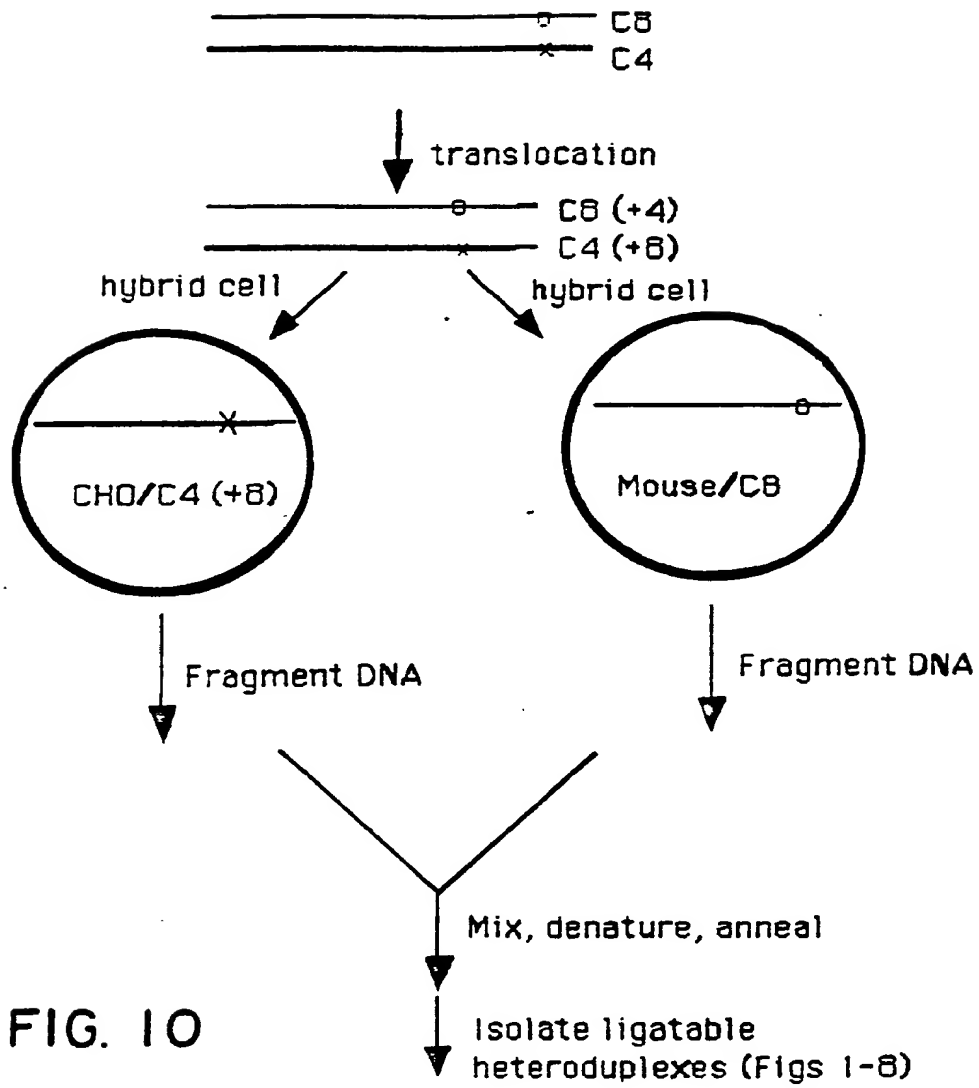

FIG. 10

Cloned telomere sequences

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02631

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int Cl. (4): C12Q 1/68

U.S. CL.: 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US.	435/6,803,172.3,253,320 436/501; 935/78 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Computer Searches: APS, Chemical Abstracts; BIOSIS, Derwent (WPI, WPIL, Biotechnology)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,Y	US, A, 4,710,465 (WEISSMAN ET AL.) 01 December 1987, see column 17, lines 18-36.	8-11,22 23
Y	Gene, Volume 24, issued September 1984 (Amsterdam, Netherlands), CHANG, S. ET AL. "Recombination following transformation of Escherichia coli by heteroduplex plasmid DNA molecules" see pages 255, 257, 259 and 260.	1-23
Y	Nucleic Acids Research, Volume 14, issued September 25, 1986 (Oxford, England) CASNA. N.J. ET AL "Genomic analysis II: isolation of high molecular weight heteroduplex DNA following differential methylase protection and Formamide-PERT hybridization" see pages 7285, 7299 and 7301.	1-23

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 October 1988	23 NOV 1988
International Searching Authority	**Signature of Authorized Officer**
ISA/US	JEREMY M. JAY

Form PCT/ISA/210 (second sheet) (Rev.11-87)

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4,594,318 (GUSELLA ET AL) 10 June 1986, see abstract.	20
Y	DNA, Volume 3, issued February, 1984 (N.Y., USA) STUDENCKI, A ET AL "Allele-specific hybridization using oligonucleotide probes of very high specific activity: Discrimination of the human beta-A- and beta-S-globin genes" see page 10.	8,22
Y	Recombinant DNA a short course, issued 1983, WATSON, J.D. ET AL., W.H. Freeman and Company (N.Y. U.S.A) see page 51.	12,13,23

BNSDOCID: <WO___8901526A1_I_>